

# **THE RELATIONSHIP BETWEEN EGGSHELL QUALITY AND BONE BIOLOGY IN THE LAYING HEN.**

By

**Sally Louise Darnell-Middleton BSc(Hons).**

Thesis submitted for the degree of Doctor of Philosophy  
in the  
Faculty of Veterinary medicine, University of Glasgow.

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#### DECLARATION

I would like to express my loving thanks to my husband Mr Scott Middleton for his patience, love and proof reading skills during the course of this project. Finally I must acknowledge the role played by my parents Mr and Mrs Roger Darnell for their unconditional love and support throughout.



Sally L. Darnell-Middleton

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1.2 Eggshell formation in the commercial laying hen

1.2.1 The egg

1.2.2 The skeletal system

I hereby declare that the work presented in this thesis was carried out by me personally with the exceptions of: Figure 2 which was prepared from electron micrographs taken by Mrs S. Cranstoun and Mr E. Lowson and Figure 3 which was adapted from the diagram published by Van de Velde *et al* (1985).

Sally L. Darnell-Middleton

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**DEDICATION**

I wish to dedicate this thesis to my husband, parents and late grandfather without whose invaluable love and support I would not have achieved my goal.

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## SUMMARY

- (1). In this thesis the relationship between eggshell quality and bone biology was examined over the laying life of two strains of hen; a highly selected commercial line and the relatively unselected J.Line.
- (2). The efficiency of using osteocalcin as a marker of osteoid formation, in collaboration with established markers of bone mineralisation and bone resorption, during eggshell calcification was ratified.
- (3). It was established that magnesium is actively removed from bone hydroxyapatite during resorption and transported to the shell gland pouch for incorporation into the palisade layer of the eggshell.
- (4). A relationship was demonstrated between hatched eggshell quality and the bone biology of newly hatched chicks.
- (5). The association between high egg output and poor bone structure was evident in the commercial laying strain hen. No such deficit in skeletal quality occurred in the relatively unselected J.Line.
- (6). The commercial line demonstrated a significantly higher rate of bone remodelling at late lay concomitant with osteoporosis.

- (7). The unselected J.Line demonstrated significantly higher rates of bone remodelling throughout lay. It was hypothesised that this was due to a greater propensity for bone upkeep during the inter-egg interval.
- (8). The provision of a prelay diet influenced neither bone volume nor mineral composition at the beginning of lay.
- (9). The feeding of a prelay diet led to a longer period of sustained high production and an earlier increase in egg weight. This was accompanied by a delay in the increase in osteoid formation and decreased levels of blood ionised calcium. This led to high "on farm" seconds at late lay due to abnormally high fracture toughness and the incidence of type B's within the mammillary layer.
- (10). The reason for the observed decrease in hatchability associated with poorly pigmented eggshells in a commercial guinea fowl flock was related to premature expulsion of the egg. These thinner shelled eggs may have resulted from the process of artificial insemination.



# LITERATURE REVIEW AND AIMS OF THESIS

## INTRODUCTION

### **CHAPTER 1**

## **LITERATURE REVIEW AND AIMS OF THESIS**

## **1 LITERATURE REVIEW AND AIMS OF THESIS**

### **1.1 INTRODUCTION**

Within commercial poultry production the hen is seen as an "entire unit" which must function optimally in order that a viable end product is achieved. However within science it is rarely possible to apply the 'entire unit' approach, thus often single aspects are studied in isolation when in fact, the inter-relationships are so complex that the study of one without the others is a gross over-simplification.

Eggshell quality is of prime importance to the commercial egg producer being the fundamental factor in the determination of egg value and thus profit. The ultrastructural eggshell quality of the commercial egg has been the subject of rigorous investigation in the last 15 years (Reid, 1984; Watt, 1985, 1989; Bain, 1990; Solomon, 1991). Interrelated with the reproductive system is the skeletal system, which supplies the calcium for eggshell formation. However, although bone status has been investigated as a welfare issue in its own right (Couch, 1955; Bell & Siller, 1962; Randall & Duff, 1988; Knowles & Broom, 1996; Wilson & Duff, 1991; Whitehead & Wilson, 1992; Wilson *et al*, 1992; Fleming *et al*, 1994; Rennie *et al*, 1997) it has rarely been investigated in association with eggshell quality.

## **1.2 EGGSHELL FORMATION IN THE COMMERCIAL LAYING**

### **HEN**

#### **1.1.1 The reproductive system**

The process of egg production in the avian commences in the left ovary where follicles are produced under the action of sex steroids and according to photoperiod. At any one time there is a follicular hierarchy with the largest follicle being the next to ovulate; approximately once every 24 hours. At ovulation the follicle ruptures along the avascularised stigma and is transported into the oviduct through the muscular actions of the infundibulum. The follicle then, in a 23 hour process, descends through the magnum (albumen-secreting region), isthmus (membrane secreting region), tubular shell gland (site of the initiation of calcification) and shell gland pouch (site of shell calcification) and thereafter through the vagina for oviposition.

In order to achieve a structurally strong eggshell it is necessary that the foundations are sound. To this end the organic components of the eggshell are vital, providing a framework within which the inorganic components are supported. The egg spends approximately 1-2 hours within the isthmus region of the oviduct during which time the tubular gland cells secrete the eggshell membranes.

The shell membranes are composed of an inner membrane surrounding the egg contents, with a distinct fine granular layer of  $2.7\mu\text{m}$  on its inner surface (Simons & Wiertz, 1963). The outer membrane, is three times as thick as the inner (Hays & Sumbardo, 1927) and integrated into the eggshell. The

composition of the two membranes is similar viz. 10% collagen, 70-75% protein and 15-20% glycoprotein, however the inner membrane contains more sialic acid and less glucosamine than the outer. Evidence suggests that the carbohydrate composition of the membrane sheaths also differs (Balch & Cooke, 1970). At the blunt pole of the eggshell the two membranes separate to form the airspace which facilitates the exchange of gas, water and minerals during incubation to the developing embryo. The membranes facilitate mechanical support and tension transmission (Simkiss, 1961), whilst they also act as a barrier to bacterial invasion (Nascimento, 1992). The membranes may also predetermine the type of calcification occurring within the eggshell. Meenaskshi *et al* (1974) demonstrated that the adhesion of avian eggshell membranes to a gap in a snail's shell resulted in avian calcite formation.

Within the isthmus, the mammillary cores are formed on the outer surface of the eggshell membranes. The substances ovokeratin (Simons & Wiertz, 1963), hydroxyproline, hydroxylysine, hexosialic acid and hexoses (Balch & Cooke, 1970), neutral mucopolysaccharides and carbonic anhydride (Robinson & King, 1968 and Robinson, 1970) have all been isolated from these sites.

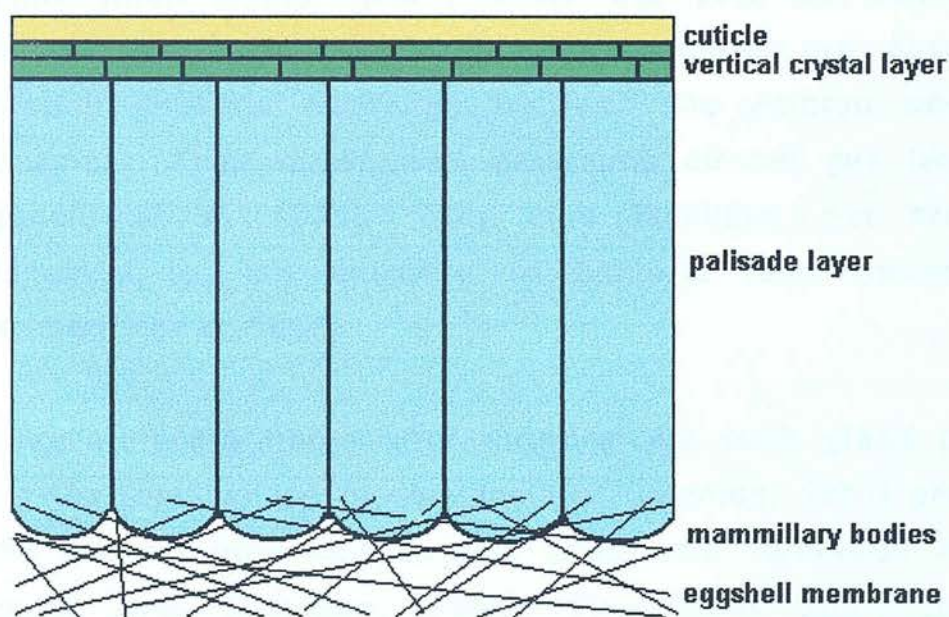
The formation of the crystalline layers commences in the tubular shell gland, with the seeding of calcium salts from the surface epithelial cells (Solomon *et al*, 1975) onto the mammillary cores to form the mammillary bodies (Taylor & Hertelendy, 1960; Robinson & King, 1968). In the shell gland pouch region, three vital processes occur, viz. plumping, matrix formation and eggshell calcification.

The first of these processes, plumping, accompanies the early stages of eggshell calcification, and is essential for the formation of a structurally sound eggshell. During a six hour process (Bradfield, 1951) plumping fluid, a combination of salts and approximately 15g of water (Burmester, 1940; Wyburn *et al*, 1973), is secreted from the tubular gland cells, via  $\text{Na}^+/\text{K}^+$  couples (Sauveur & Mongin, 1971), and absorbed into the albumen. Local pH changes in the shell gland pouch stimulate b-N-acetyl-glucosaminidase, which is synthesised in the isthmus (Tyler, 1956), to degrade  $\beta$ -ovomucin so that the albumen loses its ordered appearance (Solomon, 1979). This whole process serves to swell the egg mass and expose the seed sites necessary for the initial stages of eggshell formation.

The organic matrix is also secreted *in situ* in the shell gland pouch throughout eggshell calcification. The uterine fluid of the hen contains the same constituents as the matrix and thus may play a role in matrix formation (Nys & Gautron, 1997). The matrix is a complex of collagenous and non-collagenous protein and acid mucopolysaccharide, containing both calcium binding protein (Abatangelo *et al*, 1978) and carbonic anhydrase (Krampitz *et al*, 1974). Proteins found in the egg albumen are also present in the matrix, for example ovalbumin (Hincke, 1995). The eggshell matrix not only functions as a support structure for the inorganic constituents of the eggshell but may also regulate calcification (Arias *et al*, 1991; Boskey, 1989; Arias *et al*, 1992; Gautron, *et al*, 1996).



The eggshell consists of 95% inorganic material of which 98% is calcium carbonate, equivalent to 2.21g (Romanoff & Romanoff, 1949), 1.5% magnesium carbonate, 0.1% phosphorus oxide and approximately 0.5% aluminium and iron oxide (Almquist & Burmester, 1934). These components are transferred to the calcifying egg from the surface epithelial cells of the shell gland pouch by an  $\alpha$ -protein carrier (Solomon *et al*, 1975). The calcification of an egg takes approximately 20 hours during which time the oviducal environment and the organic matrix are responsible for the formation of three distinct crystalline layers; the mammillary, palisade and vertical crystalline layers (Simkiss, 1969, 1970; Sauveur & Mongin, 1971 & Solomon, 1991)(Figure 1).



**Figure 1:** A schematic representation of a transverse section through the eggshell

The foundation layer of the eggshell is the 100 $\mu$ m thick mammillary layer (Arias *et al*, 1993). The correct growth of the crystals in the mammillary layer is critical, not only as a foundation to the calcified shell but also in the determination of porosity and so embryonic survival.

The crystal growth patterns determine the arrangement of the mammillary bodies which in turn determine the distribution of pores. The latter are formed when adjacent mammillary bodies fail to fuse completely leading to gaps of between 0.3 and 0.9  $\mu$ m in diameter (Simkiss, 1961; Simons, 1971). The number of pores is inversely related to egg weight (Tullet, 1978). Pores may be continuous through the entire eggshell or discontinuous, as happens when crystal growth seals the pore so halting its progress through the shell. Continuous pores are essential, facilitating gaseous exchange between the embryo and the environment. Their dimensions determine air-cell gas tensions (Paganelli *et al*, 1978). They also facilitate, but are not fundamental to, the entrance of bacteria (e.g. salmonella) (Nascimento *et al*, 1992).

Approximately 5-6 hours after entering the shell gland pouch, during the rapid phase of calcification (Solomon, 1991) changes in the uterine fluid composition effect the formation of the palisade layer (Johnson, 1986). This layer 200-350 $\mu$ m in thickness (Arias *et al*, 1993), is composed of vertical columns of calcite crystals honeycombed by vesicular holes occasioned by the organic matrix. Crystal growth is uneven in this layer and ridges delimited by rhombohedral crystals can be observed on the forming front of the palisade columns (Solomon, 1991). The

palisade layer makes the largest contribution to eggshell stiffness (Bain, 1990).

The final layer of the inorganic shell is the vertical crystal layer. It consists of vertically orientated calcite crystals within a thickness of 3-8 $\mu$ m (Fujii, 1974; Arias *et al*, 1993). The change in orientation of crystals is due to a combination of pH (Solomon, 1991) and a change in the structure of the organic matrix (Fraser, 1998). The cuticle then coats the outer surface of the shell. This cuticular layer is approximately 0.5-12.8 $\mu$ m (Parsons, 1982). Secreted by the non-ciliated basal cells of the shell gland pouch (Baird *et al*, 1980) it consists of 90% peptide, polysaccharide and lipid, along with hydroxyapatite (Fraser, 1996). Constituent fractions of the cuticle and the surrounding uterine fluid from which it is formed, contain calcification inhibiting factors similar to those demonstrated in the eggshell matrix and possibly provide a mechanism for the termination of calcification (Fink *et al*, 1993). However the primary function of the cuticle is as an environmental barrier.

The pigment is deposited within the outer limits of the palisade columns and the cuticular surface of the egg. The main pigment is protoporphyrin (Baird *et al*, 1975) which is secreted from the epithelial cells lining the shell gland pouch. Distribution and patterning of the pigment is achieved through rotation of the egg by the sphincter muscle which also prevents premature oviposition (Lofts & Murton, 1973). Pigment appears to provide a final mechanical barrier, increasing crack resistance, whilst also providing cushioning to the calcite crystals of the eggshell (Solomon, 1991).



### 1.1.2 The skeletal system

The vertebrate skeleton is essentially a composite of hollow cylinders (Bell, 1959, 1969) with a tensile strength of cast iron, yet three fold lighter and ten fold more flexible (Ascenzi & Bell, 1972). The avian skeleton achieves these statistics whilst maintaining exceptionally low skeletal weight, through a combination of reduced cortical thickness, pneumatisation and the fusion or deletion of bones, as an adaptation for flight (Feduccia, 1975, King & King, 1979). Thus, the femur, tibia, ulna and radius are 10-15% less dense in birds than in mammals (Chappel, 1978). In addition, the skeletal system of the reproductively active avian also incorporates a labile mineral reservoir, medullary bone, providing storage for 89% of the body's total calcium (Martin *et al*, 1987). The process of eggshell formation is reliant on the mobilisation of this mineral.

A typical long bone is composed of a matrix of osteocollagenous fibres (Type I) in an acid mucopolysaccharide cementing substance. The bone matrix is organised into distinct lamellae or layers of 3-7 $\mu$ m thickness, within which are situated lacunae containing the bone cells. The distinct lamellae are formed due to the differences in orientation of the fibres within each layer. Canaliculi radiate from the lacunae to form a connecting pathway between individual bone cells and the vascular system. The mineral component of the bone is bound strongly within this cementing substance, in the form of hydroxyapatite ( $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ ) (Veis & Sabsay, 1987). Proteins may also be incorporated into hydroxyapatite, an example of which is osteocalcin (Hauschka *et al*, 1989).

Alterations in the proportions and arrangement of the osteo-collagenous fibres lead to the formation of the three distinct bone types (Pritchard, 1972). Cortical bone forms the dense outer cortex of a bone. It exists in three configurations which are characterised by their formation processes: i) primary osteone cortical bone which is formed as cylinders of new bone in channels within membranous bone or cartilage; ii) primary osteoid formed on the exposed periosteal or endosteal surface of existing cortical bone, usually found in small or juvenile birds (von Eggeling, 1938); iii) secondary osteone bone is formed as a result of cortical bone remodelling and is found in large adult birds such as domestic fowl and ostriches (Belanger & Copp, 1972; Currey, 1984; Rubin & Lanyon, 1988).

Trabecular bone occupies the marrow cavity in the form of narrow plates of fine lamellae, interconnecting and criss-crossing the cavity through the marrow. In female birds medullary bone occupies the marrow cavity. The surface endosteum of trabecular bone is characterised by the distinct populations of osteoblasts and osteoclasts (Pritchard, 1972; Hodges, 1974).

Cortical and trabecular bone considered together form the main structural component of the avian skeleton, however medullary bone forms the main component of the mineral reservoir for laying birds (Inoue, 1966). Found only in the marrow cavities of the long bones of reproductively active female birds it is woven in nature. Medullary bone is first formed from cortical bone (Hurwitz, 1964) prior to the onset of laying, under the influence of the sex steroids (Simkiss, 1967). The presence of medullary bone increases skeletal weight by 20%, with the greatest mass being achieved immediately prior to shell calcification (Candlish, 1971). Medullary bone, with its good vascular supply (Bloom *et al*, 1958),

and loose fibre structure, provides a readily mobilisable source of calcium, with remodelling occurring 10-15 times faster than in cortical bone (Murray & Bloom, 1948; Hurwitz, 1965). However, during periods of calcium stress medullary bone is replenished from the cortical bone (Hertelendy & Taylor, 1960; Taylor & Moore, 1954 & 1958; Candlish, 1971).

Bone is constantly being remodelled; that is at any one time bone is being resorbed, osteoid (unmineralised bone) is being formed and osteoid is being mineralised at roughly equal rates. In order to achieve these processes bone has 2 major cell types, the osteoblast and osteoclast.

Derived from primitive mesenchymal cells, osteoprogenitor cells are the stem cell precursors of osteoblasts. In mature, low turnover bone, where remodelling is minimal, osteoprogenitor cells adopt spindle forms on the bone surface. However in actively growing bone, they are much larger with oval nuclei, and are now referred to as osteoblasts. Osteoblasts are mononucleate cells with an abundance of endoplasmic reticulum, prominent Golgi apparatus and, calcium and phosphorus containing mitochondria (Bloom *et al*, 1958). Osteoblasts are responsible for the active synthesis and secretion of osteoid as mediated by parathyroid hormone (Van de Velde, 1985). Post bone formation, osteoblasts decrease in number and either become flattened or develop into osteocytes or bone lining cells (Stevens & Lowe, 1997). Both of these cell types retain a function within mature bone. Osteocytes are present in the lacunae of mineralising bone, where they are thought to maintain the organic matrix of bone and in times of calcium stress are capable of resorbing this matrix through osteocytic osteolysis. In contrast to bone lining cells they retain both the Golgi apparatus and endoreticulum of the

osteoblast and communicate via canaliculi. Bone lining cells have a decreased component of endoplasmic reticulum, ribosomes and mitochondria (Holtrop, 1990). They retain contact with the osteocytes and, in response to parathyroid hormone secretion (PTH) (1.6.2i), enzymatically dissolve the bone osteoid surface prior to resorption. Miller *et al* (1989) suggested bone lining cells may be involved in mineral homeostasis between the bone and interstitial fluid.

Osteoclasts are multinucleate cells with ample cytoplasm and vast numbers of mitochondria (Holtrop, 1990). They are thought to form from either a fusion of blood monocytes or from monocyte nuclear division (Gay, 1991). Osteoclasts function to actively resorb bone, controlled by a combination of calcium concentration and prostaglandins (Dacke *et al*, 1993). Active osteoclasts are characterised by their expansive ruffled border (Miller, 1981) whilst under hypocalcaemic conditions they lose their structure and function, with endoplasmic reticulum displaced to the periphery of the cell and cisternae distended (Zamboni Zallone & Teti, 1981).

Bone remodelling occurs constantly within all bone types. However, in the female avian this process has become specialised. Medullary bone is unique to the female where its major role is to supply the calcium required to accommodate the process of eggshell calcification. The calcium content of a single hen's egg is approximately 2.21g (Romanoff & Romanoff, 1949), thus if a figure of 300 eggs in the average commercial hen's lifetime is considered, that hen must allocate approximately 663g of calcium to eggshell production. Although 60-75% of the calcium in the eggshell is drawn directly from the food, through increased calcium uptake and digestive tract mobility (Mongin & Sauveur,

1974; Roche & Deceripit, 1977; Nys & Mongin, 1980), current housing practices mean that the laying hen is in darkness for the best part of the eggshell calcification process. Feeding does not occur during this period and although the crop may store a limited supply of food, through preferential feeding at the end of the daylight hours (Mongin & Sauveur, 1979), this is not sufficient to satisfy the requirements of eggshell calcification. The calcium deficit is filled by the skeletal system (Tyler, 1954; Bannister & Candlish, 1973; Prashad & Edwards, 1973).

In order to satisfy the demand for mineral during eggshell calcification, between 3 and 23 hours post-ovulation, only the processes of bone breakdown and osteoid formation occur. Bone mineralisation takes place between successive eggs when the demand for eggshell calcium is not present (Van de Velde *et al*, 1985). Thus, in terms of the bone cell populations, during eggshell calcification the osteoclasts actively resorb bone while the osteoblasts manufacture osteoid for mineralisation during the inactive period.

Eggshell calcification has two distinct phases (Nys, 1986). The slow phase occurs during plumping and at this time the relative numbers of bone cells are balanced and food derived calcium is present. During the rapid phase of calcification both osteoblast and osteoclast numbers increase, however osteoclast numbers exceed those of osteoblasts (Burmester *et al*, 1939; Burmester, 1940; Bloom *et al*, 1958) leading to a net bone loss. Indeed, Van de Velde *et al* (1984a) established that there was a seven fold increase in osteoclast active surface area and a two fold increase in osteoblast active surface area during eggshell calcification.



The process of bone remodelling during eggshell calcification ultimately provides a source of calcium for the eggshell. However, another component of hydroxyapatite is phosphorus which is also liberated into the bloodstream during eggshell calcification (Prashad & Edwards, 1973). The kidneys are unable to process and excrete high quantities of this mineral (Taylor & Stringer, 1965) and thus both calcium and phosphorus levels increase slowly in the first three hours of calcification (Van de Velde *et al*, 1986), before rising rapidly to a maximum around 18 hours after ovulation (Prashad & Edwards, 1973; Mongin & Sauveur, 1979). In contrast when an egg is absent from the shell gland pouch plasma calcium and phosphorus concentrations are depressed. This arises due to bone mineralisation taking place between oviposition and the entry of the next egg into the shell gland (Hughes, 1972; Sauveur & Mongin, 1974; Parsons & Combs, 1981).

Magnesium is also present within the matrix of bone. The eggshell contains approximately 20mg of magnesium (Taylor & Kirkley, 1967) located within both the mammillary cores (Itoh & Hatano, 1964) and the outer palisade layer (Brooks & Hale, 1955; Itoh & Hatano, 1964). The location of this mineral in the outer palisade layers of the eggshell would suggest a bone derived source however no research has been carried out to date characterising the fluctuating concentrations of magnesium during eggshell calcification.

The control of medullary bone remodelling during eggshell calcification is partially achieved by parathyroid hormone (PTH) (Taylor & Belanger, 1969; Prashad & Edwards, 1973). Artificial stimulation by injection of PTH, causes serum calcium levels to increase (Candlish & Taylor, 1970; Luck *et al*, 1980). Bannister &

Candlish (1973) demonstrated an increase in both medullary and cortical bone collagenolytic activity after injections of parathyroid hormone extract, the speed of which largely ruled out *de novo* synthesis of PTH. Indeed it was shown that it took 1-2 hours for PTH titres to return to their pre-depletion levels. At the bone cell level, an injection of PTH led to an increase in osteoclast area (Holtrop *et al*, 1979) and population size (Tatevossian, 1973). Miller *et al* (1984) reported that PTH administration resulted in osteoclast ruffled border development adjacent to bone surfaces after 15 minutes and endocytic vacuole appearance after 30 minutes. (Canny 1980; Taylor *et al* 1977; Weckler *et al*, 1977)

DeLuca (1983) hypothesised that PTH was stimulated by a decrease in circulating calcium during shell calcification, which in turn stimulates the hydroxylation of 25-hydroxyvitamin D<sub>3</sub> to 1,25-dihydroxyvitamin D<sub>3</sub>. DeLuca concluded this was responsible for increased intestinal absorption of calcium during the daylight hours of eggshell calcification. Van de Velde *et al* (1984b) demonstrated a rise in PTH after the commencement of shell calcification which may correspond to the observed increase in osteoclast activity. However these authors failed to demonstrate a consistent relationship between the levels of plasma ultrafilterable calcium and PTH, possibly due to the corresponding osteoblast activity. At times when there is no calcium stress, calcitonin is released to inhibit the action of PTH on the bone (Soares, 1984).

It has already been stated that increased levels of PTH stimulate dihydroxyvitamin D<sub>3</sub> (Miller *et al*, 1984; Soares, 1984; Garabedian *et al*, 1974), however it may also be stimulated by low plasma calcium concentrations directly or under the influence of sex steroids (Soares, 1984; DeLuca, 1980; Nys *et al*, 1992; Nys,

1993). Plasma 1,25-dihydroxyvitamin D<sub>3</sub> concentrations in the laying hen are double that in the prepubescent bird, correlating with the ability to produce eggshell (Soares, 1984). Moreover levels are elevated during eggshell calcification (Castillo *et al*, 1979; Spanos *et al*, 1976; Soares, 1984). 1,25-dihydroxyvitamin D<sub>3</sub> deficiency, induced by a low calcium diet (Nys *et al*, 1992), led to birds initially forming soft shelled eggs and later no eggs (Baski & Kenny, 1980). Grunder *et al* (1983) reported that hens with high levels of oestradiol produced better quality eggshells, possibly due to the stimulation of 1,25-dihydroxyvitamin D<sub>3</sub> (Kenny, 1976; Baski & Kenny, 1980; Taylor *et al*, 1971; Wecksler *et al*, 1977).

Plasma 1,25-dihydroxyvitamin D<sub>3</sub> is yet to be reliably profiled, although it is hypothesised to vary with calcium demand throughout shell formation demonstrating a peak at approximately 15 hours post ovulation (Castillo *et al*, 1979; Spanos *et al*, 1976; Soares, 1984). This is possibly due to an increase in oestrogen stimulating the renal 1 $\alpha$  hydroxylase system to produce 1,25-dihydroxyvitamin D<sub>3</sub> (Abe *et al*, 1979; Soares, 1984).

In order to reliably relate changes in eggshell quality to bone metabolism a marker of bone remodelling is required. Although techniques have been developed for assessing both PTH and 1,25-dihydroxyvitamin D<sub>3</sub>, bulk testing is not possible. Alkaline phosphatase is routinely utilised as a marker for bone remodelling in human medicine, however in the avian the majority of bone remodelling occurs during eggshell calcification when osteoid mineralisation is minimal (Taylor *et al*, 1965; Bloom *et al*, 1958).

During the search for a marker of bone remodelling in the avian Neugebauer *et al* (1995) isolated and characterised the protein osteocalcin, present within the hydroxyapatite portion of the bone



matrix. It is synthesised by vitamin-K dependant carboxylation in the osteoblasts for incorporation into the organic matrix of bone. However, some fractions, unbound to bone hydroxyapatite are released into the bloodstream (Price & Nishimoto, 1980; Delmas, 1993) from where they are detectable (Williams *et al*, 1998, Darnell-Middleton *et al*, 1998).

### **1.3 EGG SHELL QUALITY**

#### **1.3.1 The table egg**

The avian eggshell is formed according to a well defined blue print, however the nature of egg formation is such that perfection is rarely achieved. Routine grading in all commercial table egg flocks selects out the eggs demonstrating evidence of surface variation, including cracked or damaged shells, often produced in response to a stressor.

If a stress event occurs prior to the egg reaching the shell gland pouch, shell formation as a whole is affected, leading to thin or soft shelled eggs, however if eggs are in the final stages of shell deposition when the stress event occurs, shells with surface defects only are more common; for example calcium splash (dusted, lilac or pink) eggs. Such eggs demonstrate the result of retention within the shell gland pouch, with the presence of calcium phosphate spheres on the newly formed cuticle. Lilac and pink eggs are achieved due to the relationship between the cuticle and extra calcium deposits (Solomon, 1991). The duration of retention is responsible for the degrees of variation (Roberts & Brackpool, 1993-4). In extreme cases, egg retention is such that the successive egg is also present within the shell gland pouch. Due to space restriction the two eggs are forced into contact thus providing a barrier to eggshell mineralisation. In such cases the retained egg demonstrates a white band pattern, whilst the second egg becomes flattened or 'slab sided' on the contact surface. The mamillary surface of the flattened egg displays the ultrastructural variation termed confluence (Figure 2).

Adrenaline release is stimulated by a stress event, leading to the muscular contraction of the shell gland pouch. If a mineralising egg is present it may break and be subsequently repaired by additional calcification leading to a 'body checked' egg. Such eggs often show evidence of a mid line bulge with the naked eye and under candling demonstrate extensive mammillary layer cracking (Solomon, 1991).

Albumen production can also be influenced by extraneous events. In the case of corrugated eggs the albumen formed is watery, leading to uneven deposition of the eggshell membranes affecting the distribution of the calcite crystals during shell formation. This is common after exposure to Infectious Bronchitis.

The variations in quality described above are all readily observable with the naked eye or by candling techniques, usually resulting in downgrading at the packing station. Ultrastructural variations occur in the mammillary layer of the eggshell and, although they may coincide with surface variation, are routinely found in eggshells which have passed normal quality controls. The mammillary layer forms the foundations for the rest of the eggshell thus it is not surprising that variations within this layer have ramifications within the shell as a whole. At present, there are 12 documented ultrastructural variations in the mammillary layer (Figure 2) which have been scored according to their perceived influence on egg performance in the field (Reid, 1984; Watt, 1985, 1989; Bain, 1990; Solomon, 1991; Fraser, 1996). These modifications together with measures of the effective and mammillary thickness (Bain, 1990) provide a complete assessment of the structural quality of any one egg.

**Figure 2:** Ultrastructural variations in the mammillary layer of the eggshell of the hen (Courtesy of Poultry Science Unit, University of Glasgow).

**Cap Quality** Caps (NM) should demonstrate adequate coverage of the mammillary body and a surface pitted with deep channels on plasma etching .

**Fusion** This term describes the growth of adjacent palisade columns. If the latter fuse early then the effective thickness of the shell is increased and *vice versa*.

**Confluence** Confluence (CON) occurs when mammillary bodies are no longer spatially discrete, influencing the distribution of pores and the formation of the palisade layer. This condition, frequently found in eggs from young birds and in slab sided eggs, may lead to areas of weakness.

**Changed membrane** The sulphated groups associated with the membrane fibres are a small but significant part of their composition; in the normal egg, plasma etching removes these groups along with the ash of the organic layer. However, many eggs examined after plasma treatment still possess an abnormally sulphur rich mat of "changed membrane" adhering to the mammillary layer.

**Alignment** Alignment relates to the ordered (or otherwise) spatial arrangement of the mammillary surface. Aligned mammillae offer a low crack resistance.

**Type A bodies** These can appear singly or in groups. Their contact with the membrane fibres is minimal and they have a conical appearance at the level of the mammillary layer. Growth of the cone and palisade layer does occur but the columns so formed rarely contribute to the entire thickness of the shell.

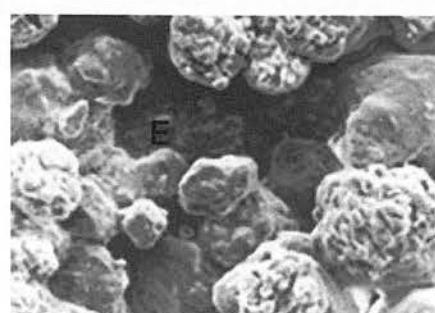
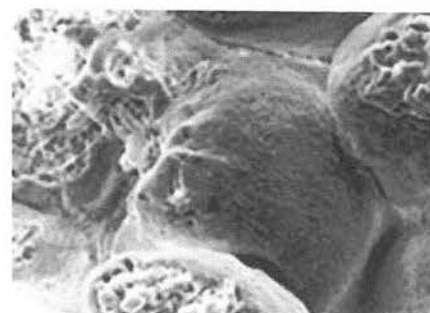
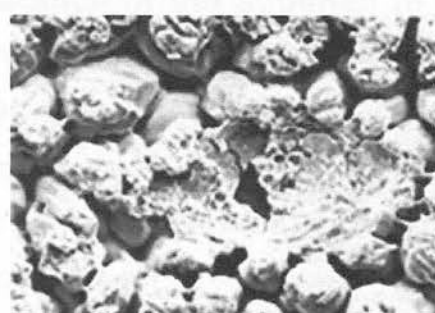
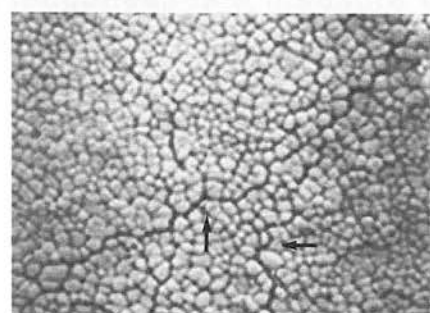
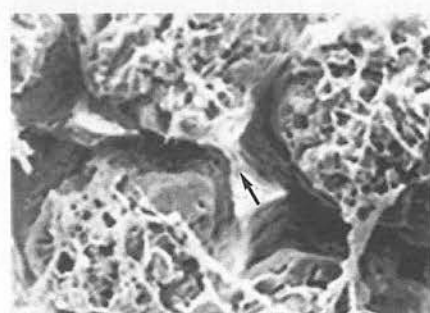
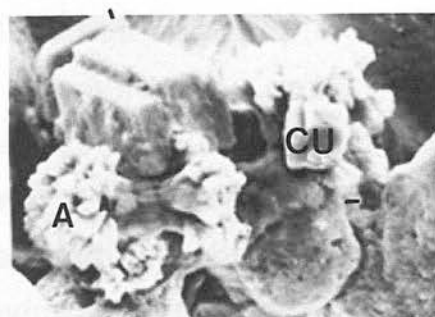
**Cubics & Aragonite** Given available space calcium carbonate will grow preferentially as a cubic form. When the formation of normal palisade columns is impaired, individual cubic crystals (CU) frequently occupy intermammillary sites. Aragonite (A) is the preferential form of calcium carbonate in reptilian eggshells. Variations from the norm do occur in nature with aragonite sometimes being present in the avian eggshell and calcite in the reptilian.

**Cuffing** Extracrystalline cuffs (C) at the junction of the cone and palisade layers assist in the early fusion of the palisade columns and thereby enhance the shells resistance to cracking (Bain, 1990).

**Type B bodies** Rounded type B bodies occur more frequently in the mammillary layer than type A bodies. Although they make contact with the membrane fibres to varying degrees, they make no contribution to the true thickness of the shell.

**Depression** This refers to areas which display concave distortion to the normal mammillary layer appearance. They arise through the accumulation of oviducal debris on the shell membranes, which in turn either interferes with the distribution of nucleation sites, or masks their presence.

**Erosion** This feature relates to pitting of the mammillary surface to varying depths. Erosions (E) typically lack normal basal cap and cone structure thus exposing the underlying palisade layer.





The analysis of eggshell ultrastructure has been routinely used in the detection of stress within commercial poultry flocks. Brackpool (1995) demonstrated the feasibility of the technique as a non-invasive method of assessing the welfare of laying hens. Watt (1989) demonstrated that stress induced by both adrenaline and stocking density influenced oviducal structure and function within the oviduct and hence lead to the production of structurally imperfect eggs for a period of 14 days post event. Research has been conducted demonstrating the effects of age (1.4), diet (1.5), genotype (1.6), environment (Mohamed, 1986; Fraser & Bain, 1994; Nascimento, 1992; Brackpool, 1995), moulting (Brackpool, 1995) and both the temperature and salinity of drinking water (Brackpool, 1995) on ultrastructural eggshell quality.

The role of traditional measurements of eggshell quality is documented within the review by Hunton (1995). In addition Bain (1990) pioneered the use of the material property, fracture toughness in the analysis of eggshell strength. Fracture toughness is a measure of the resistance of the eggshell to crack growth taking into account the nature and magnitude of any defects within the shell. Bain (1990) concluded that the structural modification termed late fusion led to a decline in the fracture toughness of an eggshell, however other modifications such as cap quality had little effect on this parameter.

### 1.3.2 The hatching egg

Despite both an industry and public focus on table eggs the fact remains that the prime role of the eggshell is as an embryonic chamber, from which the developing chick must gain 80% of its calcium requirements (Simkiss, 1961), whilst also fulfilling the roles of protection and gaseous exchange. However despite these requirements, the fact remains that eggshell quality in hatching eggs has received little attention with all uncracked eggs entering the incubator.

The twelve ultrastructural variants which are evident within table eggs can also be observed in eggs destined for the incubator, although their effects differ due to the final role of the shell. Thus the structural modifications entitled early and late fusion and cuffing will alter the effective thickness of the eggshell and hence the ease with which the embryo can pip the shell. It is also possible to assess at the ultrastructural level the extent of calcium uptake from the mammillary bodies in newly hatched shells. Although Roberts *et al* (1992) commented on the persistence of certain variants at hatch, they made no attempt to score shell quality using these characteristics.

Within the eggshell, foetal bone development occurs by the complementary processes of intramembranous and endochondral ossification which lead to the formation of a complete skeletal system prior to hatching (Gay, 1988). The calcium content of the embryo increases in three distinct periods at 1-8, 9-15 and 15-21 days (Simkiss, 1961; Johnson & Comar, 1955). The initial period of calcification of the embryo appears to be supported by the yolk, as calcium transport from the shell to the developing chick does not commence until day 10-12 of embryonic development.



The movement of calcium between the eggshell and chick reaches a maximum around day 19 (Tuan & Scott, 1977) corresponding to the attainment of normal plasma levels of 1,25-dihydroxyvitamin D<sub>3</sub> (Kubota *et al*, 1981). Bone resorption also occurs within the embryonic chamber. PTH is secreted by day 10 of development (Narbaitz, 1972) and osteoclasts are recognisable (Khan *et al*, 1981) so that by day 12-13 calcium resorption from the long bones is possible (Ramp & McNeil, 1978; Rosen *et al*, 1980). However it is thought that osteoclasts only operate with true efficiency from day 19 (Martini *et al*, 1982) which corresponds with the normal titre of 1,25-dihydroxyvitamin D<sub>3</sub> being reached.

#### **1.4 THE EFFECTS OF AGE ON EGGSHELL QUALITY AND THE INTEGRITY OF THE SKELETAL SYSTEM**

The external defects and ultrastructural variations of the avian eggshell have been discussed in some detail. Although the majority of these variations can occur randomly as the result of a stress event, the nature of modern poultry production is such that many other factors influence eggshell quality. One of the major factors acknowledged to result in changes in eggshell quality is age.

Age related changes in the eggs of laying hens are well recognised. Older birds produce larger eggs due to a lower number of follicles reaching maturity and thus an increase in the amount of yolk per egg. Production (Boorman *et al*, 1985; Bain, 1990), quality in general (Boorman *et al*, 1985; Watt, 1989; Bain, 1990; Nascimento, 1990; Roberts & Brackpool, 1993-4) and shell strength (Brooks, 1971; Watt, 1989; Bain, 1992) all decrease with increasing age. Watt (1989) showed that eggs became more abnormal at the level of the mammillary layer as bird age increased, although the poorest "on farm" seconds were achieved at early lay. The most likely explanation for these changes with age is the lack of correlation between shell weight and egg size (Washburn, 1982; Brackpool *et al*, 1993), thus it is assumed as egg weight increases with age there is no concomitant increase in shell weight, therefore leading to thinner shells. Johnson (1986) also commented on a decrease in membrane thickness with age.

Creel *et al* (1998) demonstrated chick quality problems from early and late lay parent stock. The age of the parent stock was also found to affect incubation time, egg weight at transfer and chick weight at hatch (Suarez *et al*, 1997).

Both at the time of formation of medullary bone (Thorp, 1994) and during the laying life of the commercial hen, structural bone is used to replenish medullary bone reserves (Taylor & Dacke, 1984; Thorp *et al*, 1993; Knott *et al*, 1995). Petersen (1965) demonstrated that a decrease in the available mineral within the laying hen skeleton may have a role to play in the decrease in eggshell quality observed at late lay, as there is no decline in the ability to absorb or mobilise calcium with age (Roland *et al*, 1975; Roland, 1980). Approximately 5% of all British layers (Randall & Duff, 1988) and 15-30% of all United States layers (Roland & Rao, 1992) are osteoporotic by the time of slaughter. The discrepancy between the results being due to the culling of the British flock after one term of lay. The high incidence of osteoporosis is not only important from a welfare point of view but also from the commercial aspect as many of the spent hen carcasses are unsalable because of bone splinters remaining in the deboned meat (Gregory & Wilkins, 1989).

Avian osteoporosis was first described by Couch (1955). However Bell & Siller (1962) published the first detailed pathological description of both the acute and peracute forms of the disease. These authors found cortical thinning of the long bones whilst a highly calcified medullary bone was present. An excess of osteoclasts was present in both bone types. There were no differences in the concentration of plasma total calcium, magnesium and inorganic phosphate. The authors tentatively suggested that the decrease in bone strength was due to the

affected birds lacking a mechanism to shut off their parathyroid hormone release when calcium levels were low. Thus medullary bone, in osteoporotic birds, is produced at the expense of cortical bone (Taylor & Moore, 1954).

More recently Wilson *et al* (1992) demonstrated the progressive nature of structural bone loss throughout the term of lay, with a figure of 11% trabecular bone volume being indicative of severe osteoporosis (Whitehead & Wilson, 1992). These authors demonstrated a lack of trabecular bone osteoid and osteoblasts in osteoporotic hens, although both were present within the medullary bone (Taylor *et al*, 1971). Further, Hudson *et al* (1993) showed through fluorochrome labelling of mature laying hens, that mineralisation occurred solely in the medullary portion of the bone. Thus it can be hypothesised that the basis of the pathology of osteoporosis is an imbalance in the remodelling of structural bone to favour resorption. Knott *et al* (1995) demonstrated clear changes in the collagen matrix of osteoporotic bone, possibly initiated in an attempt to repair the latter but which, in fact lead to an acceleration in the fragility of the bone matrix.

Osteoporosis is a particular problem in commercial egg laying flocks. Age has a significant effect upon the relationship between housing and the incidence of this disease (Singsen *et al*, 1969; Fleming *et al*, 1994). King (1975) correlated size of cage with severity of symptoms. The weaker bones in such birds may be due to a lack of activity leading to a increase in structural bone loss accompanied by a decrease in bone strength (Wilson *et al*, 1992; Fleming *et al*, 1994). Similar symptoms were found following immobilisation of one leg in a plaster cast (Pilaski, 1970). After Perrins (1992) identified osteoporosis in hens as a priority for welfare research, further study was carried out on this

subject area (Whitehead & Wilson, 1992; Wilson *et al*, 1993) with the result that no significant correlation with cancellous bone volume was found in birds with different exercise and housing regimes. The addition of perches to cages did serve to increase cancellous bone volume but not to decrease the incidence of osteoporosis. Thus it was concluded that further, as yet unidentified, factors were ultimately responsible for the development of osteoporosis in laying hens.

Rennie *et al* (1997) attributed the incidence of avian osteoporosis to the length of continuous reproduction to which the modern commercial hen is subjected. Nevertheless, there exists no current literature correlating both avian osteoporosis and the acknowledged deficits in eggshell quality occurring at the end of lay (Bain, 1990).

Genotypical differences have been observed in shell strength (Bowman & Challenger, 1963; Potts & Washburn, 1974; Potts & Waxenberg, 1963), thickness (Tyler & Giese, 1958; Bowman & Challenger, 1963; Potts & Washburn, 1974), shell weight per unit of surface area (Hamilton, 1976), specific gravity (Potts & Washburn, 1974; Hamilton, 1976) and egg weight (Marron *et al*, 1983). Suarez *et al* (1997) demonstrated significant effects of exercise on laying performance.



## **1.5 GENOTYPICAL DIFFERENCES IN EGGSHELL QUALITY AND BONE REMODELLING**

Genetic variation within the flocks of different companies is such that it is possible to select for the improvement of individual egg characteristics (Buss, 1982), thus it is to be expected that there will exist genotype differences in eggshell quality (Buss, 1982; Solomon, 1990). Recent selection has focused on specifics such as shell colour and thickness. However a more rounded approach must be utilised as eggshell thickness *per se* does not necessarily reflect better performance in the field. The heritability of egg characteristics, such as egg weight and egg density is greater than for egg production traits (Besbes *et al*, 1992), thus poor egg quality may be a trait inherited as a by-product of selection for increased production. Similarly Fleming *et al* (1996) demonstrated that humeral and tibial strengths and keel radiographic densities were positively correlated with body weight implying that selection for production parameters without controlling for body weight could lead to the incorporation of deleterious bone traits into the genotype.

Genotypical differences have been observed in shell strength (Bowman & Challender, 1963; Potts & Washburn, 1974; Potts & Washburn, 1983), thickness (Tyler & Geake, 1958; Bowman & Challender, 1963; Potts & Washburn, 1974), shell weight per unit of surface area (Hamilton, 1978), specific gravity (Potts & Washburn, 1974; Hamilton, 1978) and egg weight (Marion *et al*, 1964). Suarez *et al* (1997) demonstrated significant effects of strain on hatching performance.

Thorp (1994) states that "selection pressure for production traits in modern lines of poultry has placed increasing demands on skeletal integrity". Rennie *et al* (1997) demonstrated significant differences between the skeletal histomorphometry of the J.Line, a relatively unselected Brown leghorn strain, and the Hisex Brown at late lay. The J.Line, having a lower egg output did not experience the structural bone loss during lay attributed to the Hisex Brown. It has still to be determined whether the absence of skeletal defects in the J.Line is mirrored by the maintenance of good eggshell quality throughout lay.

## **1.6 THE EFFECTS OF DIETARY MANIPULATION ON EGGSHELL QUALITY AND BONE REMODELLING**

Nutrition is the essential component of any poultry system. The egg gains its quota of calcium carbonate from both diet directly and indirectly from the medullary bone system which is sustained via the diet. The major components in the diet of the laying hen reflect these needs with calcium and phosphorus making up a large proportion of the mineral composition of the diet (Appendices 1 & 2).

Dietary induced calcium stress results in a reduction in the rate of egg production (Hurwitz & Bar, 1969) and eggshell thickness (Hurwitz & Bar, 1969; Washburn, 1982; Narbaitz *et al*, 1987). The method of diet presentation can also have an effect with particulate diets leading to stronger shells, through higher plasma concentrations of calcium and lower concentrations of phosphorus (Guinotte & Nys, 1991). However, it is important to achieve a suitable balance as although phosphorus can be found in small quantities throughout the eggshell (Tyler, 1954), high phosphorus levels, through the diet or forced bone remodelling (1.6.3), lead to negative effects on eggshell formation (Simkiss, 1969; Miles *et al*, 1983; Sauveur & Mongin, 1983). 1,25 dihydroxy-vitamin D<sub>3</sub> is one of the major regulators of bone remodelling during eggshell formation, and so it is no surprise that diets deficient in this vitamin can also lead to a decrease in egg production and shell thickness (Ikeme *et al*, 1983; Narbaitz *et al*, 1987). The feeding of vitamin D metabolites to older hens has been demonstrated to result in an increase in shell strength (Morris *et al*, 1977). The time of feeding may also affect eggshell quality. Harms *et al*



(1996) reported that the midnight feeding of laying hens led to quality improvements in eggs laid the following morning.

Magnesium is also present as a trace element in layer diets, however research on its exact functional role is sparse. Anon (1967) suggested it may have a role in the maintenance of shell strength. Magnesium is deposited as magnesium carbonate (Romanoff & Romanoff, 1949), primarily in the outer layers of the eggshell (Itoh & Hatano, 1964; Brooks & Hale, 1955). Magnesium, like phosphorus, has the ability to substitute for calcium in bone, thus in periods of low calcium it can be hypothesised that an increase in magnesium would also occur both in the bloodstream and in the eggshell. Robinson (1987) hypothesised that magnesium may also replace lysozyme in ovomucin in order to stabilise some components of albumin. Interestingly, Albeck *et al* (1993) commented on the effect of magnesium on the crystal structure of calcium in sea urchin spines, with a magnesium:calcium ratio of above 4:1 leading to the formation of aragonite crystals.

In terms of the skeletal system, Riddle *et al* (1968) and Antillon *et al* (1977) looked at the effects of a low-phosphorus or low calcium diet respectively, and found thinned and easily broken bones with both. Deformed sternum and ribs were common, as were compression fractures involving the fourth or fifth thoracic vertebrae, leading to paralysis. The pathology of the bone, in these cases was identical to that of confirmed osteoporotic birds (Schiefer & Dorn, 1969). Similar, yet less severe symptoms were found with vitamin D deficiency (Antillon *et al*, 1977; Wilson & Duff, 1991). Again enlargement of the parathyroid glands was found in deficient birds (Antillon *et al*, 1977), thus adding weight to Bell & Sillers's (1962) hypothesis that affected birds lacked a

mechanism to shut off their parathyroid hormone release when bone calcium levels were low. No significant effects on the incidence of osteoporosis have been demonstrated as a result of dietary calcium supplementation (Rennie *et al*, 1997).

The modern commercial layer diet exists in different stages according to the age of the hen, for example starter and grower diet, prelay diet, layer diet 1 and layer diet 2. These diets correspond to the stages of bird development, growth, medullary bone formation and lay respectively. In terms of the relationship between eggshell quality and bone remodelling one of the most vital times in bird development is the time of medullary bone remodelling. At approximately 14-16 days prior to the onset of lay medullary bone is formed, under the actions of androgen & oestrogen (Taylor & Stringer, 1965) from the remodelling of structural bone. The theory behind the incorporation of the prelay diet is that dietary calcium supplementation at this time will result in more available calcium for this process and thus benefits in long term bone structure. Hurwitz (1964) demonstrated that the addition of supplementary calcium during medullary bone formation results in its incorporation within the structural component of bone, however a trial at Harper Adams Agricultural College (1991) failed to demonstrate any significant effects on bone strength. Inconsistent results have also been recorded on the relationship between the feeding of a prelay diet at 16 weeks and the incidence of egg quality characteristics (Harper Adams Agricultural College, 1990).

## 1.7 EGG SHELL QUALITY IN AN EXOTIC SPECIES:

### THE GUINEA FOWL

Thus far in the introduction attention has been directed towards the role of the eggshell as a hatching chamber for the domestic fowl. This role has to be satisfied by all egg laying species. Failure to hatch in the wild is common place and is often compensated for by large numbers of eggs in any one clutch. In the commercial situation failure to hatch represents a financial loss and as more exotic species reach the supermarket shelf every effort must be directed towards maximising hatchability.

The guinea fowl eggshell is thicker than that of the laying hen as a result of an increase in the linear phase of eggshell deposition (Panheleux *et al*, in press British Poultry Science). It is surprising to observe that the increased duration of eggshell formation in the guinea fowl is not indicated by any increase in the interval between ovipositions. According to Panheleux (1999a) the rapid phase of shell deposition begins earlier. Consistent with the observation that the histological pattern of the shell gland pouch is more compact than that of the domestic fowl (Panheleux, 1999a), it is not surprising to observe differences in the nature and volume of the proteins secreted as contributions to the organic matrix. Panheleux (1997) has demonstrated differences in the matrix protein pattern and had hypothesised that such changes contribute to the increased breaking strength observed in these shells (Petersen & Tyler, 1967; Sauveur, 1988). Under "normal" conditions the same range of structural variations exists as previously described in the laying hen (Panheleux *et al*, 1999b).

Most birds and reptiles have the ability to retain their eggs beyond the normal time of oviposition and as a consequence display many shell surface defects. In the layer bird these shells are automatically downgraded. Overtly splashed eggshells in the domestic fowl are rarely set for hatching. This is not the case with species such as the guinea fowl, perhaps because of the low egg numbers every attempt is made to hatch all eggs regardless of appearance. The splashed eggs laid by the guinea fowl have a poor prognosis, nevertheless no evidence exists to date as to the reasons underlying such failure to hatch.

The techniques for the ultrastructural analysis of eggshell quality are well documented for table eggs. The second aim of this thesis was to establish if such techniques could be translated to the analysis of successfully hatched eggshells (Chapter 3) and if any relationship is apparent between hatched eggshell quality and newly hatched chick bone mineral content.

Low hatchability and high chick mortality are widely acknowledged in the guinea fowl. In the penultimate chapter of this thesis (Chapter 5) the techniques for assessing eggshell quality were applied to the more exotic "table" species to establish the basis for chick mortality in one specific case study associated with shell colour.

## **1.9 AIMS OF CURRENT WORK**

It is clear from the existing literature that as a direct result of the increased demand on the commercial layer, the incidences of poor eggshell quality and osteoporosis have increased. However traditionally the fields of eggshell quality and bone histomorphometry have been treated as separate entities. The prime aim of this thesis was to integrate the accumulated knowledge from both fields to investigate the relationships between them. With regard to this aim, the first priority was to develop and test a method of assessing medullary bone formation (Chapter 2). In both chapters 3 and 4 this technique was utilised, along with more established methods, to ascertain the changes in both eggshell quality and bone histomorphometry with age, genotype and feeding regimes in laying hens.

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## AN EVALUATION OF OSTEOCALCIN AS A MARKER FOR BONE TURNOVER IN THE LAYING HEN.

### 2.1 INTRODUCTION

## **CHAPTER 2**

### **AN EVALUATION OF OSTEOCALCIN AS A MARKER FOR BONE TURNOVER IN THE LAYING HEN.**

## 2 AN EVALUATION OF OSTEOCALCIN AS A MARKER FOR BONE TURNOVER IN THE LAYING HEN.

### 2.1 INTRODUCTION

The specialised extracellular matrix of bone, termed osteoid, is a collagenous support tissue in which the collagen, designated Type I, is embedded in a glycoprotein rich gel, a major component of which is osteocalcin (Hauschka *et al*, 1989). Other proteins have also been identified namely osteonectin and osteopontin. The sequential mineralisation of the osteoid gives bone its characteristic rigidity.

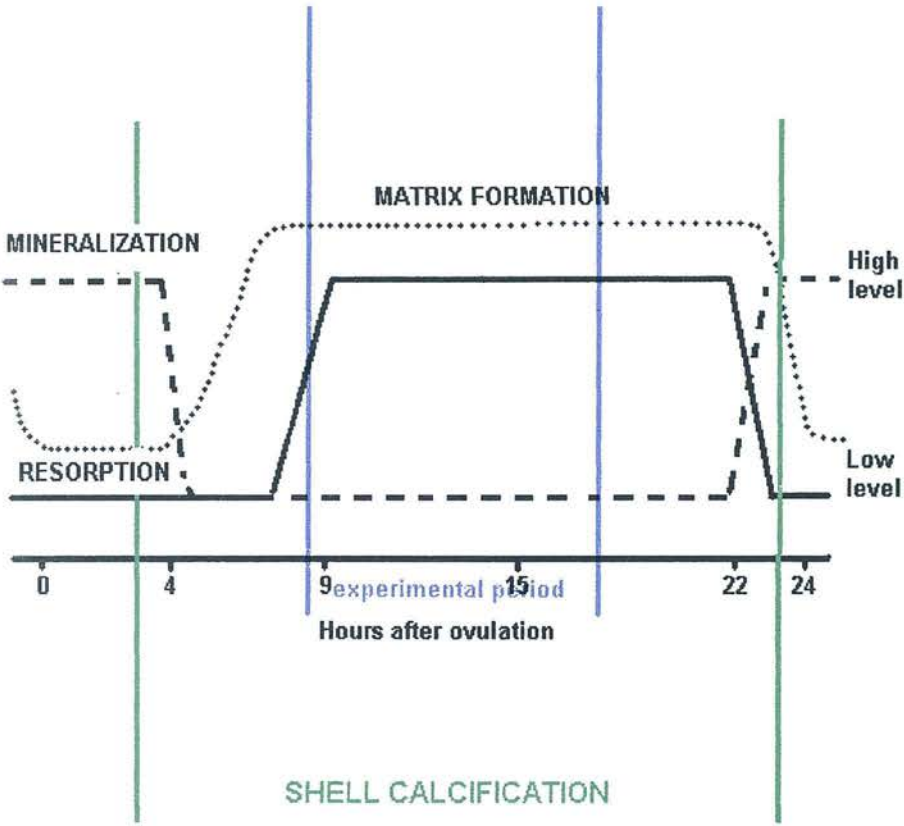
Bone is a dynamic tissue in which hormonal and physical factors interact to alter its volume and deposition in response to physical stress. Such re-organisation is not species specific, thus in young children bone turnover is high, thereafter it declines to the *status quo*, but can increase from its normal basal level to meet unforeseen demands such as fracture repair. In birds the situation is more complex. In addition to the demands of skeletal support, bone is implicated in the process of shell calcification, and to that end medullary bone plays a major role (see 1.1.2).

Bone remodelling, that is the processes of formation and resorption, is mainly under the control of two cell types; osteoblasts and osteoclasts (see 1.1.2). Osteoblasts are responsible for the production and release of osteoid. As these primitive cells differentiate from the osteoprogenitor cells, so they demonstrate sequential expression for alkaline phosphatase, osteonectin, osteopontin and osteocalcin. Osteoclasts, in

contrast, characterised by their multinucleate appearance and ruffled border are the mediators of bone resorption.

In the hen, eggshell calcification occurs between 3 and 23 hours postovulation, with a peak at 18 hours, during which time the food related calcium is rapidly exhausted leading to bone remodelling (Candlish, 1971a). Van de Velde *et al* (1985) used cellular data to establish a model for bone remodelling during one laying cycle in the hen (Figure 3). Approximately 1 hour after the start of eggshell calcification these authors found a decrease in medullary bone mineralisation, accompanied by an increase in bone matrix formation, which was confirmed by Wilson & Duff (1990). Accompanying these changes Van de Velde *et al* (1984b) also demonstrated a decrease in plasma ultrafilterable calcium which stimulated an increase in parathyroid hormone (PTH) at 6 hours postovulation. PTH (1.1.2) is responsible for osteoclast activation (Miller *et al*, 1984) and thus, at 7/8 hours post ovulation and 5/6 hours after the start of eggshell calcification, bone resorption is initiated. Phosphorus is present within the mineralised bone matrix, incorporated into hydroxyapatite ( $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ ), and is released into the circulation during bone resorption (Mongin & Sauveur, 1979). Thus, the concentration of inorganic phosphorus in the plasma increases from 10 hours post ovulation (Mongin & Sauveur, 1979; Sauveur & Mongin, 1983) due to the inability of the kidney to process and excrete such elevated quantities of this mineral (Feinberg *et al*, 1937; Taylor & Stringer, 1965; Prashad & Edwards, 1973; Parsons & Combs, 1981; Watanabe & Ishibashi, 1993).





**Figure 3:** Model of the processes of resorption, matrix formation, and mineralisation of the medullary bone during the egg-formation cycle. As postulated by Van de Velde *et al*, 1985.

According to Van de Velde *et al* (1984b) PTH levels are minimal at 22 hours postovulation, while total plasma calcium concentration is decreasing (Paul & Snetsinger, 1969; Mueller *et al*, 1973). These findings correspond with a decrease in the rate of shell calcification (Burmester, 1940; Sauveur & Mongin, 1971) and the time at which the outer layers of the proteinaceous cuticle are laid down. At 23 hours post ovulation shell calcification is complete, and at this time there is a decrease in bone resorption as revealed by the decreasing plasma phosphorus concentration (Sauveur & Mongin, 1983). The newly secreted bone matrix is mineralised in the time between the end of calcification of the preceding egg and the transit into the shell gland pouch of the following egg (Van de Velde *et al*, 1985).

While plasma PTH concentration could be used as a reliable indicator of bone turnover, suitable large scale assays are not yet available. Alkaline phosphatase (see 1.1.2) assay kits, on the other hand, are commercially available and have been applied in both human and veterinary medicine to monitor bone turnover (Moss, 1992). Released by the active osteoblast, alkaline phosphatase breaks down the crystalline inhibitor pyrophosphate in vertebrate blood plasma (Moss, 1992) during osteoid mineralisation (Magnusson *et al*, 1995; Yamamoto *et al*, 1995). However, according to Figure 3 alkaline phosphatase concentration is elevated outwith the period of eggshell calcification (Taylor *et al*, 1965; Bernard *et al*, 1980). Moreover alkaline phosphatase is released by other organs such as the liver and this further complicates the interpretation of the results (Delmas *et al*, 1983; Gevers *et al*, 1986; Delmas & Malaval, 1987; Deftos, 1991; Parviainen *et al*, 1991; Delmas, 1992; Delmas, 1993). Although separation of bone associated alkaline phosphatase is possible (Rennie, 1994) it is a time consuming

and expensive procedure, making this a less than satisfactory method for the monitoring of changes during bone remodelling in the laying hen.

Osteocalcin (bone  $\gamma$ -carboxy-glutamic acid protein) (1.1.2) is synthesised by vitamin-K dependant (Hauschka & Reid, 1978a) carboxylation in the osteoblasts and to a lesser extent odontoblasts (tooth building cells) (Lian & Friedman, 1978; Nishimoto & Price, 1980). Thereafter it is incorporated into the extracellular matrix (Romberg *et al*, 1986). However some fractions, unbound to the matrix are released into the bloodstream (Price & Nishimoto, 1980; Price *et al*, 1981; Lian & Gundberg, 1988; Vanderschueren *et al*, 1990; Delmas, 1993). In this situation it is thought to serve as a negative regulator of bone matrix formation (Ducy *et al*, 1996; Wolfe, 1996) viz. if bone matrix formation increases above its physiological optimum, the resultant increase in osteocalcin concentration in the bloodstream inhibits further bone matrix formation (Menanteau *et al*, 1982; Boskey *et al*, 1985; Romberg *et al*, 1986). In the human, plasma osteocalcin has been used to detect altered bone metabolism in metabolic bone disorders (Power & Fottrell, 1991).

In the avian, osteocalcin is released uniquely from the osteoblasts during osteoid formation (Figure 3) (cf. alkaline phosphatase). Boskey *et al* (1985) confirmed that osteocalcin is not involved during bone mineralisation, suggesting that alkaline phosphatase and osteocalcin are related to different bone activities (Chenu *et al*, 1990). Although osteocalcin is incorporated into the hydroxyapatite component of medullary bone, it is not released into the circulating blood during bone resorption (Price *et al*, 1981). These factors make osteocalcin an ideal marker of bone matrix formation (Hauschka *et al*, 1975;

Price *et al*, 1976; Delmas & Malaval, 1987; Delmas, 1993). Osteocalcin was characterised in the avian by Neugebauer *et al* (1995) and a suitable assay has since been developed (Bouillon *pers comm*).

Osteocalcin synthesis is stimulated by 1,25 dihydroxy-vitamin D<sub>3</sub> (see 1.1.2) (Price & Nishimoto, 1980; Markowitz *et al*, 1987; Carpenter *et al*, 1992). Nys *et al* (1986) demonstrated a significant increase in 1,25 dihydroxy-vitamin D<sub>3</sub> at 4 hours postovulation, rising to a maximum at 9 hours postovulation. It thereafter remained at a constant level. These findings are consistent with the start of bone matrix formation and thus osteocalcin production.

Carpenter *et al* (1992) demonstrated a decrease in plasma osteocalcin in magnesium deprived rats, suggesting a possible relationship between these two parameters. They also reported decreases in bone volume associated with disorganised bone remodelling in their experimental animals and hypothesised a role for magnesium in the development of osteoporosis. Within the laying hen magnesium is present in both the mineralised bone and the eggshell. In the bone, magnesium ions are incorporated into the interior of the hydroxyapatite crystals. The eggshell contains approximately 20mg of magnesium (Taylor & Kirkly, 1967) distributed between the mammillary cores (Itoh & Hatano, 1964) and outer palisade layer (Brooks & Hale, 1955; Itoh & Hatano, 1964). To date only Taylor (1961) has attempted to characterise circulating magnesium levels during eggshell mineralisation.

Brooks & Hale (1955) demonstrated a higher absorption of calcium and magnesium in the laying hen (Taylor & Kirkly, 1967). Taylor & Kirkly (1967) demonstrated a higher absorption of calcium and magnesium in the laying hen (Taylor & Kirkly, 1967). Taylor & Kirkly (1967) demonstrated a higher absorption of calcium and magnesium in the laying hen (Taylor & Kirkly, 1967).

The formation of the eggshell, during each laying cycle, places considerable demand upon the bone and in so doing relies primarily on the maintenance of the dynamic equilibrium that exists between the bone cell populations. Selection for increased egg numbers in the modern commercial layer has detrimentally influenced this *status quo* as evidenced by the reported elevated levels of osteoporosis at the end of lay (Gregory & Wilkins, 1989 & 1992) and the subsequent decline in eggshell quality with age (Bain, 1990; Nascimento, 1990; Nascimento *et al*, 1992, Roberts & Brackpool, 1993-4). To date it has been difficult to monitor the bone related changes which occur during the main phase of eggshell calcification due to the lack of a suitable assay for bone matrix formation (Figure 3). The aim of this chapter was therefore to compare the newly available assay for osteocalcin with established methods for observing bone turnover in the laying hen.

This chapter begins with a demonstration of osteocalcin concentrations during one laying cycle. The effects of removing the calcifying egg prematurely, at 8 hours postovulation, on blood related parameters, including osteocalcin, were then investigated. It was hypothesised that this action would lead to a subsequent decrease in bone matrix formation and hence an increase in bone mineralisation (Figure 3).

It has been documented that a low calcium diet in laying hens alters the balance of osteoclast and osteoblast cell populations (Zamboni Zallone & Teto, 1981). This results in a poorly calcified bone matrix (Bernard *et al*, 1980) with an elevated phosphorus and magnesium content (Taylor & Moore, 1956). Hurwitz & Bar (1966 & 1969) demonstrated a higher absorption of calcium from the bone matrix during eggshell calcification in



calcium deprived birds, suggesting that the birds under dietary deprivation had higher plasma inorganic phosphate concentrations. Bloom *et al* (1958) demonstrated an increase in osteoid formation under similar conditions. These findings were substantiated by Nys *et al* (1992), who reported an increase in osteocalcin in hens fed on a low calcium diet for 2 weeks. In the final section of this chapter, the methodologies applied above are used to investigate the influence of feeding a low calcium diet to laying hens for 24 hours. Given that calcium deprivation will usually go undetected for only a minimal time under commercial conditions, the aim of this experiment was to establish if such a short period of calcium deprivation had a notable effect on bone turnover.

### 2.2.2 BLOOD SAMPLES

Blood samples of approximately 5ml were taken from the wing vein of the right wing of the birds at 2, 11, 14 and 17 hours following oviposition. Blood was centrifuged for 5 minutes at 2000 g and 0.2 ml aliquots of the plasma stored at -20°C prior to carrying out the assay. The plasma inorganic phosphate assay, on the other hand, was carried out within 12 hours of fresh plasma (Nys *et al*, 1992).



## **2.2 MATERIALS & METHODS**

### **2.2.1 ANIMALS**

Three groups of 8, 45 week old, commercial laying hens were selected at random from a flock maintained on standard layer mash (*Nys pers comm*) and a 14L:10D lighting regime. Time of oviposition was monitored to the nearest minute by a trip-switch on the cage connected to a recording device. Group 1 hens were maintained as control birds. Group 2 hens were maintained on a control diet but received an injection of Dinoprost tromethane-Dinolytic ( $\text{PGF}_{2a}$ ) (Laboratoire UPJOHN) ( $50\mu\text{g}/10\mu\text{l}$  0.9% NaCl) at 8 hours post oviposition to induce premature expulsion of the next egg. Group 3 hens were placed on a wheat only diet (*Nys pers comm*) 24 hours prior to experimentation, thus enforcing low calcium conditions. Both Group 1 and Group 3 hens received a placebo injection of NaCl at 8 hours post oviposition. Experimentation only took place on hens which were in mid-sequence due to the differences in shell related parameters demonstrated in the early and late eggs of a sequence (Belyavin *et al*, 1985).

### **2.2.2 BLOOD SAMPLES**

Blood samples of approximately 5ml were taken from the wing vein, in heparinised syringes, at 8, 11, 14 and 17 hours following oviposition. Blood was centrifuged down for 5 minutes at 3000 g and 0.5ml aliquots of the plasma stored at  $-20^{\circ}\text{C}$  prior to carrying out the assay. The plasma inorganic phosphorus assay, on the other hand, was carried out within 12 hours on fresh plasma (*Nys pers comm*).

**i) Osteocalcin.** Plasma samples were subjected to radioimmunoassay for osteocalcin, using pure chick osteocalcin and the specific antibody KOC1 (Bouillon, R., University of Louvain, Belgium). Standards were prepared according to the protocol of Bouillon (*pers comm*) utilising the previously described chick osteocalcin. All samples were diluted tenfold prior to the assay because of the high levels of circulating protein (Williams *pers comm*). Antibody was diluted 1:2000,000 and  $^{125}\text{I}$ -osteocalcin labelled using the chloramine-T method (Bolton, 1985). 100ml of standard/sample and 100ml of antibody were incubated overnight at 4°C. A further overnight incubation at 4°C followed the addition of 100ml  $^{125}\text{I}$ -osteocalcin (10,000 c.p.m.). Separation of bound from free antibody was achieved by the addition of sheep anti-rabbit serum (1:20) and incubation for 2 hours at 4°C. A final 30 minute incubation took place after the addition of 1ml of 0.4% PEG 6000 and 0.1% Tween 20 mixture. Samples were then centrifuged at 3000g for 30 min, before decanting and counting the remaining sample in a gamma spectrophotometer (Packard Instruments, Meriden, CA, USA) for 1 min. Note that there were no control plasmas included in this assay as there is no universally accepted control plasma for chicken osteocalcin in existence (Williams *pers comm*).

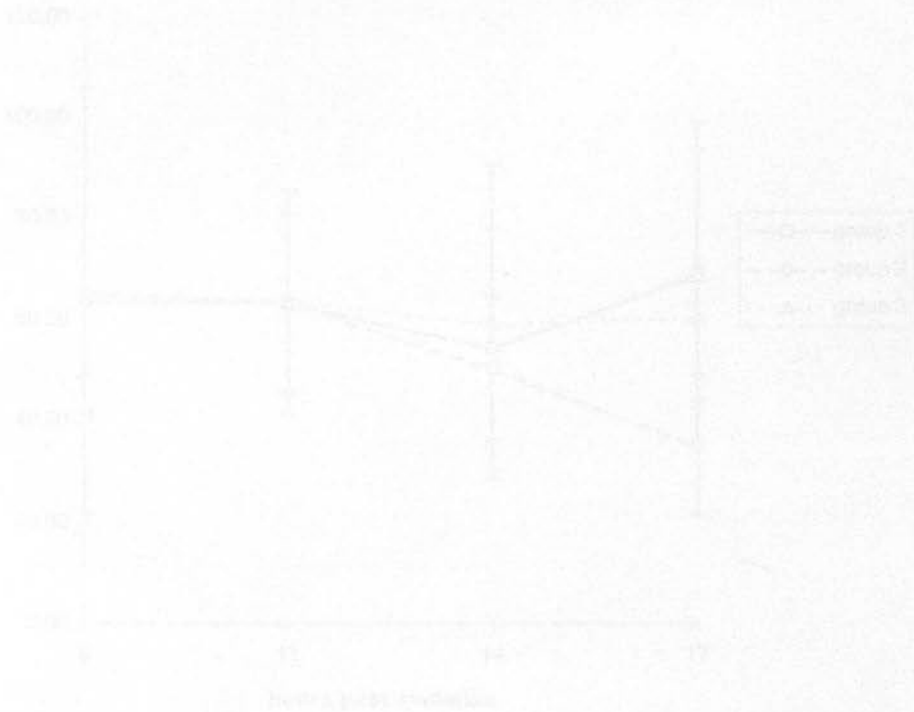
**ii) Established biochemical assays.** 1,25 dihydroxyvitamin D<sub>3</sub> levels were measured using a Gamma-B 1,25 dihydroxyvitamin D kit (IDS, Boldon, Tyne on Wear, England). Samples were diluted two fold. Alkaline Phosphatase was estimated using a Biotrol Alkaline Phosphatase kit, modified for use with avian plasma and an automatic plate reader. In this case plasma was diluted with distilled water by a factor of 1:10 (Whitehead *pers comm*), due to the higher concentrations of alkaline phosphatase in laying hen plasma. In all cases both standards and controls were supplied with the kit. Calcium and magnesium were measured using Wako kits (Alpha Laboratories, Hampshire) modified for use with avian plasma and for measurement with an automatic plate reader (Titertek Twin-Reader Plus, ICN)(Whitehead *pers comm*). The circulating concentrations of these biochemicals is also higher than those in humans and thus plasma must be diluted twofold in the case of calcium (Rennie *et al*, 1997) and four fold for magnesium (Darnell-Middleton unpublished data) with distilled water. Control values did not vary from those expected from kit measurements. Plasma inorganic phosphate was evaluated after dialysis using a Technicon autoanalyser.

### **2.2.3 DATA ANALYSIS**

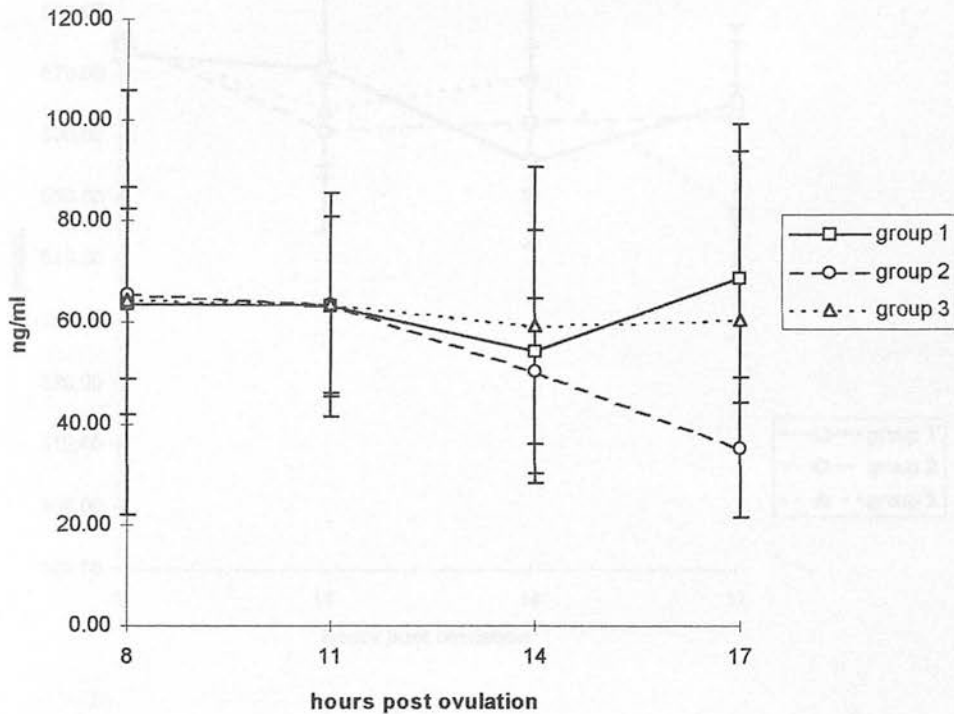
The data were analysed using the Minitab statistical package (Release 11; Minitab Inc, Pasadena, USA) and inserted into a general linear model (glm) to look at group|time interactions. A post-hoc t-test was subsequently carried out on all significant data. The mean and standard deviation of the mean were also calculated and represented graphically.

## 2.3 RESULTS

The means and standard deviation of the means corresponding to each time period for Groups 1 and 2, and Groups 1 and 3, are presented along with the results of the general linear model (glm) for group|time interaction and the post-hoc t-tests in Tables 1 and 2 respectively. All data are represented graphically in Figures 4-9. Summaries of the results of all the blood related parameters measured within each group, between 8 and 17 hours post ovulation, are given in figures 10-12.

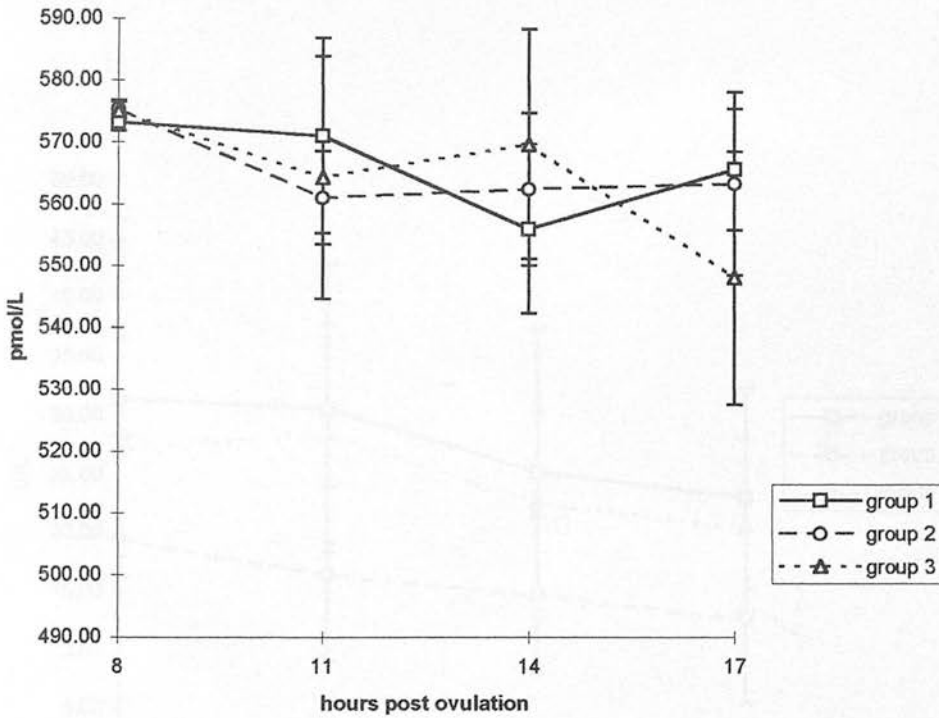


**i) Osteocalcin.** There was no significant change in the mean plasma concentration of osteocalcin in Group 1 (control) with hours postovulation. In Group 2 (expulsed) the mean plasma osteocalcin concentration displayed a significant decrease between 11 and 17 hours post-ovulation. There was a statistically significant difference between Group 1 and Group 2 at 17 hours post ovulation. In Group 3 (low calcium) there was no significant change in the mean plasma concentrations of osteocalcin over time.



**Figure 4:** Mean osteocalcin concentration between 8 and 17 hours post ovulation, incorporating standard deviation of the mean.

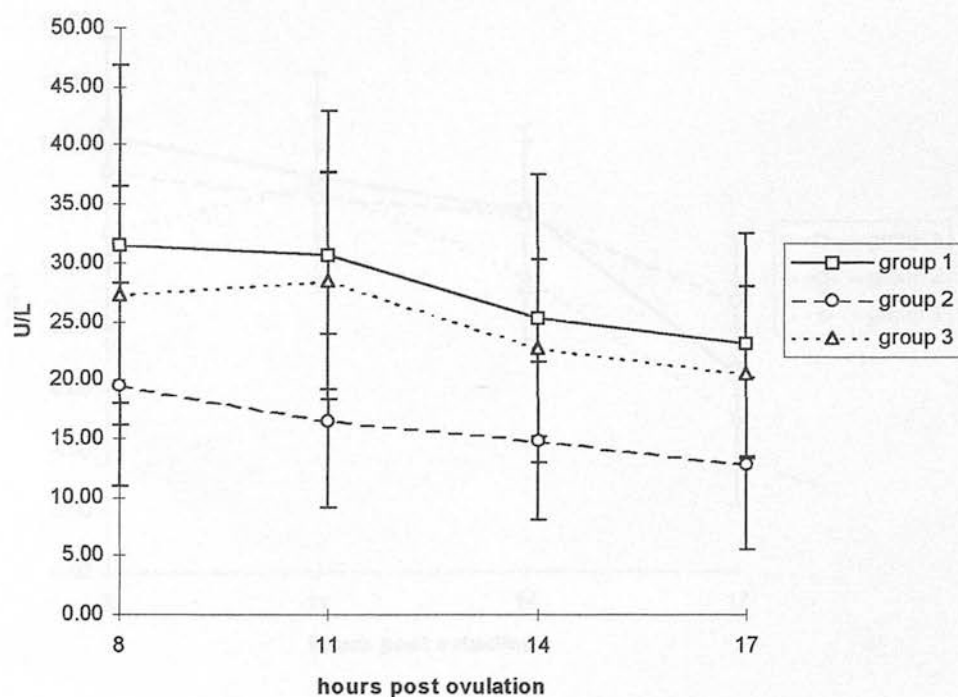
ii) **1,25 dihydroxy-vitamin D<sub>3</sub>.** The mean plasma 1,25 dihydroxy-vitamin D<sub>3</sub> concentrations showed no significant variation with time either within or between groups. However, Group 3 displayed a trend towards a significant decrease in mean 1,25 dihydroxy-vitamin D<sub>3</sub> concentration between 14 and 17 hours postovulation.



**Figure 5:** Mean 1,25 dihydroxy-vitamin D<sub>3</sub> concentration between 8 and 17 hours post ovulation, incorporating standard deviation of the mean.

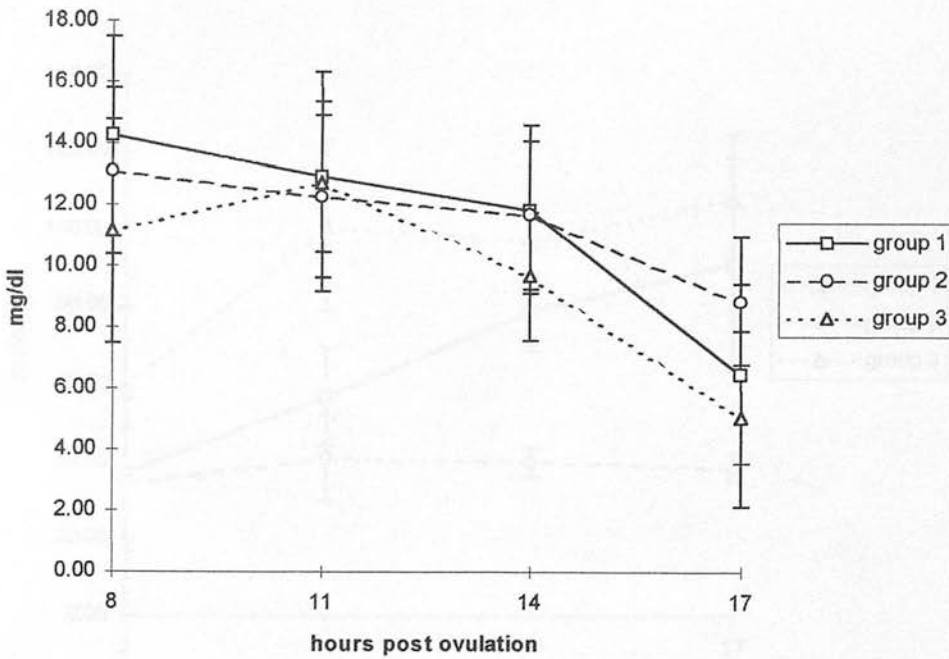


**iii) Alkaline Phosphatase.** Mean plasma alkaline phosphatase concentration remained constant over time in Group 1. Group 2, however, demonstrated a lower mean plasma alkaline phosphatase concentration throughout but this was only significant between 8 and 11 hours post ovulation (cf. Group 1). A more critical examination of the data revealed a large range in alkaline phosphatase concentrations in Group 2. In Group 3 mean plasma alkaline phosphatase concentration remained constant over time and did not show any significant difference with respect to Group 1 (control).



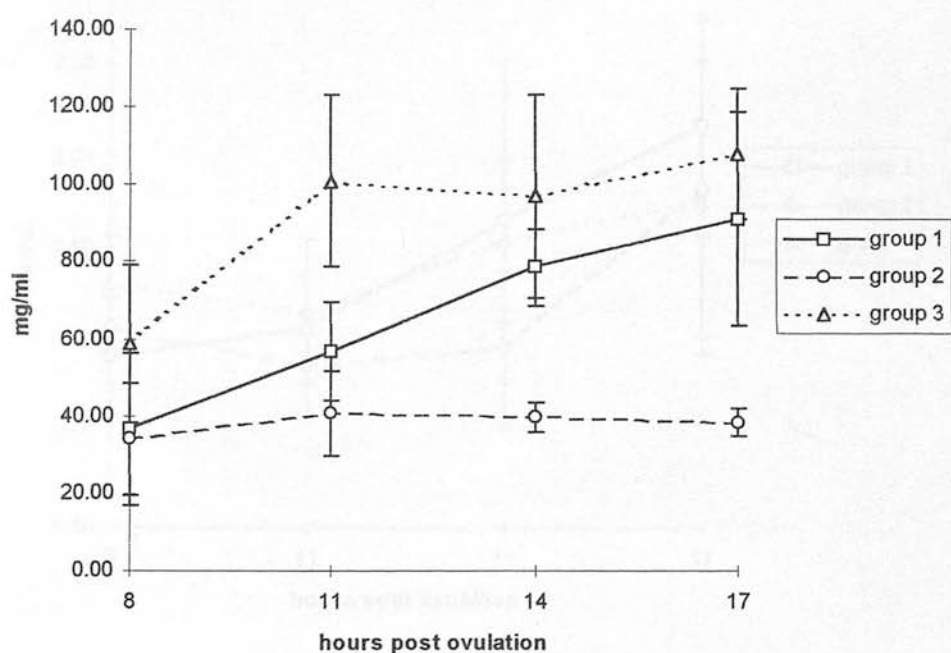
**Figure 6:** Mean alkaline phosphatase concentration between 8 and 17 hours post ovulation, incorporating standard deviation of the mean.

**iv) Total Calcium.** Mean plasma total calcium concentration decreased significantly between 14 and 17 hours post-ovulation in Group 1. Group 2 showed a similar pattern with time but the decrease observed between 14 and 17 hours was of a lower magnitude. There were no significant differences in plasma calcium concentrations between Group 2 and Group 1. In Group 3 mean plasma total calcium concentration decreased significantly from 11 hours post ovulation. At 8 hours post ovulation Group 3 had significantly lower mean plasma total calcium concentrations than Group 1.



**Figure 7:** Mean total calcium concentration between 8 and 17 hours post ovulation, incorporating standard deviation of the mean.

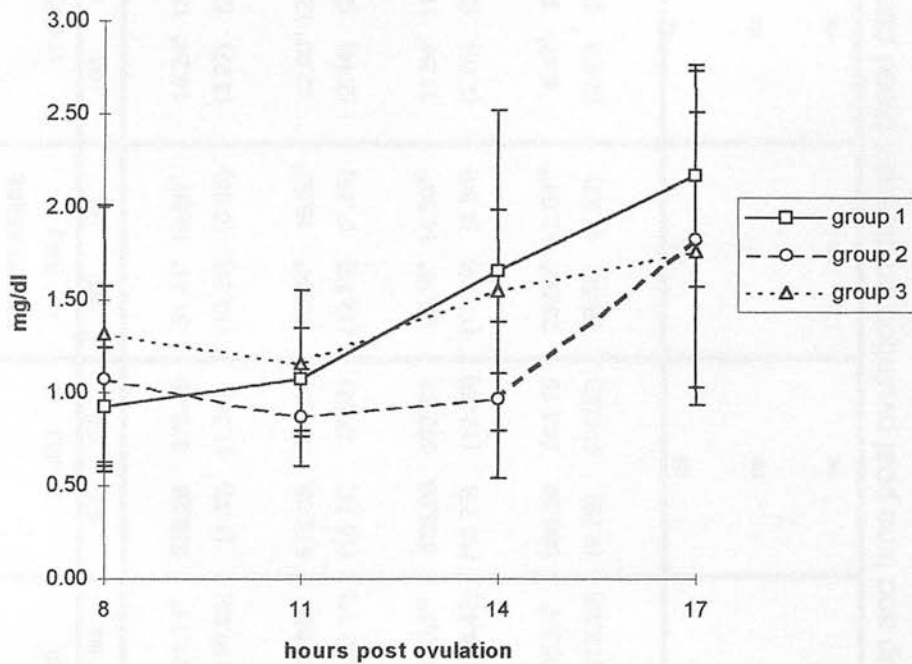
**v) Inorganic phosphate.** Mean plasma inorganic phosphate increased significantly between 8 and 11 hours post ovulation and between 11 and 14 hours post-ovulation in Group 1. In Group 2 the mean plasma inorganic phosphate remained constant throughout the experiment and was significantly less than Group 1 at 11 hours, 14 hours and 17 hours post-ovulation. In Group 3 mean plasma inorganic phosphorus increased significantly between 8 and 11 hours post-ovulation, thereafter they remained fairly constant. At 8 and 11 hours postovulation Group 3 displayed significantly higher concentrations of inorganic phosphate, than Group 1.



**Figure 8:** Mean inorganic phosphate concentration between 8 and 17 hours post ovulation, incorporating standard deviation of the mean.

**vi) Magnesium.**

Between 11 and 14 hours post ovulation the mean plasma magnesium in Group 1 increased significantly. The levels remained elevated until the end of the experimental period. An increase in mean plasma magnesium also occurred in Group 2 but in this case not until between 14 and 17 hours post ovulation. Thus the mean plasma magnesium concentration was significantly higher in Group 1 at 14 hours post ovulation. In Group 3 the pattern of concentration change with time was similar to that observed in Group 1, although the changes were not considered significant.



**Figure 9:** Mean magnesium concentration between 8 and 17 hours post ovulation, incorporating standard deviation of the mean.

TIME (hrs post ovulation)	OSTEOCALCIN (ng/ml)		VITAMIN D3 (pmol/L)		ALKALINE PHOSPHATASE (U/L)		TOTAL CALCIUM (mg/dl)		INORGANIC PHOSPHATE (mg/ml)		MAGNESIUM (mg/dl)	
GROUP	G1	G2	G1	G2	G1	G2	G1	G2	G1	G2	G1	G2
8	63.84 <sup>a</sup> (41.90)	65.71 <sup>a</sup> (16.66)	573.20 (1.38)	575.45 (1.38)	31.44 <sup>a</sup> (15.29)	19.56 <sup>b</sup> (8.65)	14.24 <sup>a</sup> (3.27)	13.08 <sup>a</sup> (2.71)	36.62 <sup>a</sup> (19.79)	34.13 <sup>a</sup> (14.65)	0.93 <sup>a</sup> (0.32)	1.08 <sup>a</sup> (0.50)
11	63.22 <sup>a</sup> (17.77)	63.65 <sup>a</sup> (22.11)	571.03 (15.77)	561.00 (7.53)	30.64 <sup>a</sup> (12.33)	16.53 <sup>b</sup> (7.38)	12.90 <sup>a</sup> (2.44)	12.25 <sup>a</sup> (2.66)	56.63 <sup>b</sup> (12.72)	40.75 <sup>a</sup> (10.87)	1.07 <sup>a</sup> (0.28)	0.87 <sup>a</sup> (0.26)
14	54.24 <sup>a</sup> (24.09)	50.35 <sup>ab</sup> (14.42)	555.90 (13.75)	562.33 (12.39)	25.18 <sup>a</sup> (12.29)	14.80 <sup>ab</sup> (6.80)	11.84 <sup>a</sup> (2.76)	11.65 <sup>a</sup> (2.40)	78.62 <sup>c</sup> (9.90)	39.75 <sup>a</sup> (3.77)	1.66 <sup>b</sup> (0.86)	0.97 <sup>a</sup> (0.42)
17	68.87 <sup>a</sup> (24.77)	35.24 <sup>b</sup> (13.86)	565.50 (9.79)	563.15 (14.82)	23.00 <sup>a</sup> (9.57)	12.84 <sup>ab</sup> (7.32)	6.47 <sup>b</sup> (2.92)	8.86 <sup>b</sup> (2.08)	90.75 <sup>c</sup> (27.55)	38.38 <sup>a</sup> (3.46)	2.17 <sup>b</sup> (0.60)	1.83 <sup>b</sup> (0.90)
TIME	ns		ns		ns		**		**		**	
GROUP	*		ns		**		ns		**		ns	
GROUP TIME	*		ns		ns		ns		**		ns	

**Table 1:** Influence of group and time post ovulation on different blood parameters in the laying hen.

( ), standard deviation of the mean; a-c, means with same superscript do not differ significantly ( $p < 0.05$ ); G1, control group; G2, expulsed egg group;

\*\*  $p < 0.01$ ; \*  $p < 0.05$ ; ns not statistically significant.

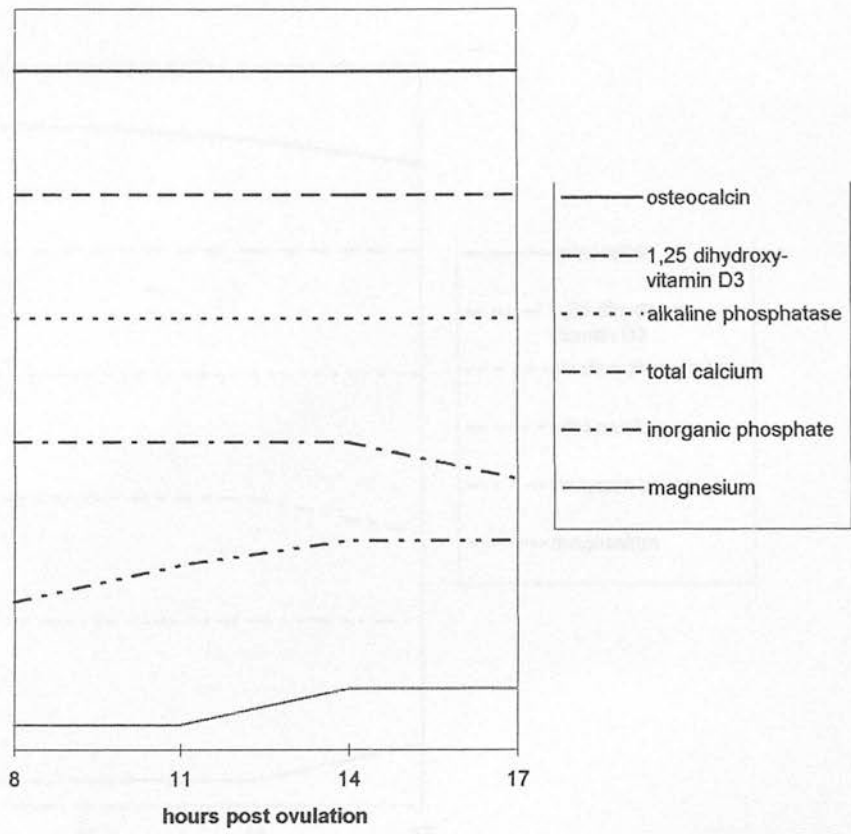
TIME (hrs post ovulation)	OSTEOCALCIN (ng/ml)		VITAMIN D3 (pmol/L)		ALKALINE PHOSPHATASE (U/L)		TOTAL CALCIUM (mg/dl)		INORGANIC PHOSPHATE (mg/ml)		MAGNESIUM (mg/dl)	
GROUP	G1	G3	G1	G3	G1	G3	G1	G3	G1	G3	G1	G3
8	63.84 (41.90)	64.39 (22.40)	573.20 (1.38)	575.10 (1.38)	31.44 (15.29)	27.28 (9.19)	14.24 <sup>a</sup> (3.27)	11.13 <sup>cd</sup> (3.63)	36.62 <sup>a</sup> (19.79)	58.86 <sup>b</sup> (20.41)	0.93 <sup>a</sup> (0.32)	1.32 <sup>ab</sup> (0.69)
11	63.22 (17.77)	63.56 (17.35)	571.03 (15.77)	564.25 (19.65)	30.64 (12.33)	28.44 (9.31)	12.90 <sup>a</sup> (2.44)	12.74 <sup>ac</sup> (3.56)	56.62 <sup>a</sup> (12.72)	100.86 <sup>c</sup> (22.03)	1.07 <sup>a</sup> (0.28)	1.16 <sup>a</sup> (0.39)
14	54.24 (24.09)	59.48 (31.29)	555.90 (13.75)	569.58 (18.54)	25.18 (12.29)	22.72 (7.65)	11.84 <sup>ad</sup> (2.76)	9.67 <sup>d</sup> (2.09)	78.62 <sup>bc</sup> (9.90)	97.00 <sup>c</sup> (26.31)	1.66 <sup>b</sup> (0.86)	1.55 <sup>ab</sup> (0.44)
17	68.87 (24.77)	60.47 (38.90)	565.50 (9.79)	547.98 (20.35)	23.00 (9.57)	20.58 (7.36)	6.47 <sup>b</sup> (2.92)	5.01 <sup>be</sup> (2.86)	90.75 <sup>bc</sup> (27.55)	107.86 <sup>c</sup> (16.92)	2.17 <sup>b</sup> (0.60)	1.77 <sup>b</sup> (0.74)
TIME	ns		ns		ns		**		**		**	
GROUP	ns		ns		ns		*		**		ns	
GROUP TIME	ns		ns		ns		ns		ns		ns	

**Table 2:** Influence of group and time post ovulation on different blood parameters in the laying hen.

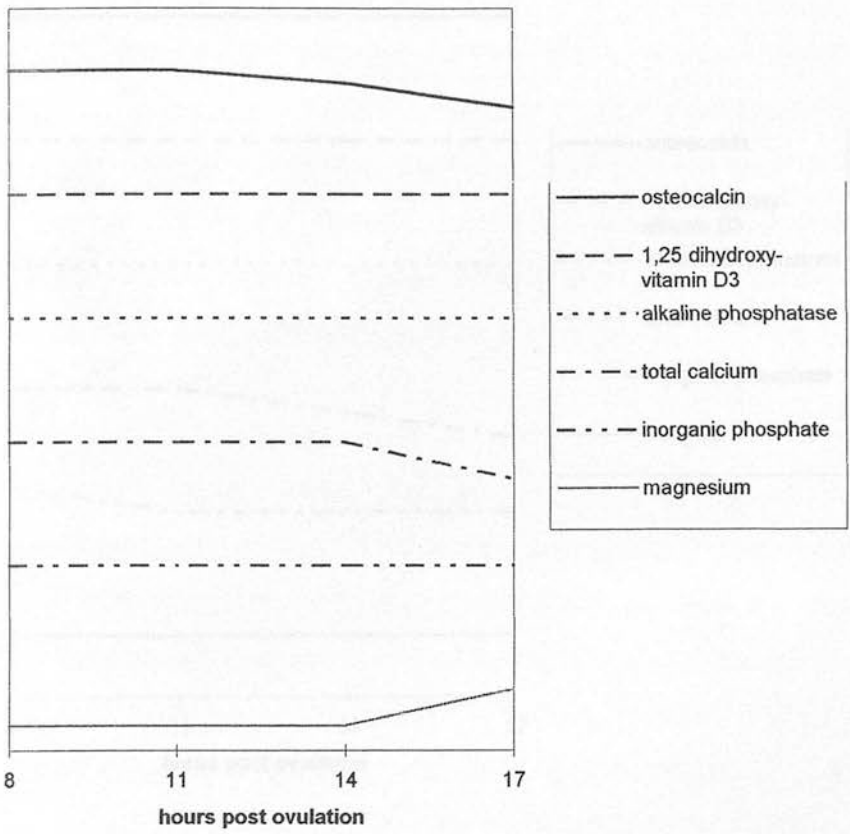
( ), standard deviation of the mean; a-e, means with same superscript do not differ significantly ( $p < 0.05$ ); G1, control group; G3, low calcium group;

\*\*  $p < 0.01$ ; \*  $p < 0.05$ ; ns not statistically significant.

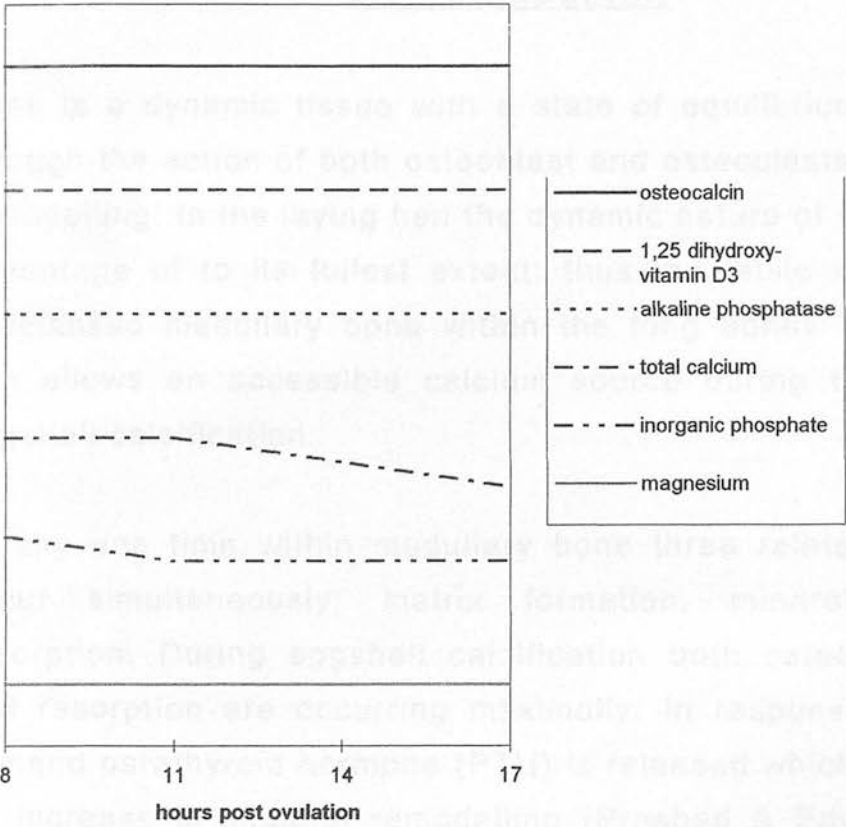




**Figure 10:** Summary of results for the control group (Group 1).



**Figure 11:** Summary of results for the expelled egg group (Group 2).



**Figure 12:** Summary of results for the low calcium group (Group 3).

## 2.4 DISCUSSION

Bone is a dynamic tissue with a state of equilibrium maintained through the action of both osteoblast and osteoclasts during bone remodelling. In the laying hen the dynamic nature of bone is taken advantage of to its fullest extent; thus the labile nature of the specialised medullary bone within the long bones of the laying hen allows an accessible calcium source during the period of eggshell calcification.

At any one time within medullary bone three related processes occur simultaneously; matrix formation, mineralisation and resorption. During eggshell calcification both osteoid formation and resorption are occurring maximally. In response to calcium demand parathyroid hormone (PTH) is released which encourages an increase in skeletal remodelling (Prashad & Edwards, 1973) through the stimulation of 1,25-dihydroxyvitamin D<sub>3</sub> (Miller *et al*, 1984; Soares, 1984; Garabedian *et al*, 1974). This leads to a seven fold increase in osteoclast active surface area and a two fold increase in osteoblast active surface area (Van de Velde *et al*, 1984a). Price (1985), Markowitz *et al* (1987) and Carpenter *et al* (1992) observed an increase in osteocalcin synthesis in association with the increase in osteoblast activity. Osteocalcin is synthesised by vitamin K dependant carboxylation (Hauschka & Reid, 1978) in the osteoblast (Lian & Friedman, 1978; Nishimoto & Price, 1980) for incorporation into the extracellular matrix of bone (Romberg *et al*, 1986). The unbound fractions of the protein are released into the bloodstream (Price & Nishimoto, 1980; Price *et al*, 1981; Lian & Gundberg, 1988; Vanderschueren *et al*, 1990; Delmas, 1993) where, as reported in the introduction, they serve as negative regulators of bone matrix formation (Ducy *et al*, 1996;

Wolfe, 1996). The assay for osteocalcin provides a measure of these fractions.

This chapter set out to investigate the changes in osteocalcin occurring between 8 and 17 hours postovulation when, according to Figure 3, there is a period of relatively stable bone dynamics with respect to matrix formation, resorption and mineralisation. This also corresponds to the rapid phase of eggshell calcification (Nys, 1986). A summary of the results of this experiment are presented in Figures 10-12.

The results presented in Table 1, Figures 4 and 10 clearly demonstrate that the mean plasma osteocalcin concentration remained constant over the study period, inferring that, in Group 1, the rate of bone matrix synthesis was constant. The concentrations of osteocalcin compare favourably to those generated by Nys (1993), with any minor differences being due to individual labelling for assay runs (Williams *pers comm*).

When the calcifying eggshell is prematurely removed from the shell gland pouch (Group 2), at 8 hours post ovulation, a decrease in osteocalcin concentration, relative to the control group, was evident from 11 hours post ovulation. Thus the removal of the calcifying egg has, in a matter of only 3 hours, led to a decline in bone matrix formation.

The effects of imposing low calcium conditions have been well documented. Hypocalcaemia leads to a poorly calcified bone matrix (Bernard *et al*, 1980) with an elevated phosphorus and magnesium content (Taylor & Moore, 1956). During eggshell calcification this led to higher rates of resorption (Hurwitz & Bar, 1966 & 1969) and matrix formation (Bloom *et al*, 1958).

According to Figures 4 and 12 however, when birds are fed a low calcium diet for only 24 hours (Group 3) their mean plasma osteocalcin concentrations were unaffected. Thus a short period of hypocalcaemic conditions does not appear to have any effect on the rate of bone matrix formation in the laying hen.

Plasma 1,25 dihydroxy-vitamin D<sub>3</sub> concentrations in the laying hen are known to be double that in the prepubescent bird and correlate with the ability to produce eggshell (Soares, 1984). Although it has been observed that 1,25 dihydroxy-vitamin D<sub>3</sub> levels are elevated during eggshell calcification (Castillo *et al*, 1979; Spanos *et al*, 1976; Soares, 1984), they are yet to be reliably profiled (Nys *pers comm*). The production of osteocalcin is stimulated by 1,25 dihydroxy-vitamin D<sub>3</sub> (Price, 1985; Markowitz *et al*, 1987; Carpenter *et al*, 1992), with increasing concentrations occurring between 4 and 9 hours post ovulation (Nys *et al*, 1986).

According to the results of this study the mean concentration of 1,25 dihydroxy-vitamin D<sub>3</sub> was constant in the control birds between 8 and 17 hours post ovulation, which is in agreement with Nys *et al* (1986) and so in the light of this evidence there was no stimulus for a change in the rate of bone matrix formation; thus explaining the constant concentrations of osteocalcin.

When the calcifying egg was removed from the shell gland pouch (Group 3) at 8 hours postovulation the concentration of 1,25 dihydroxy-vitamin D<sub>3</sub> again did not change. This is in marked contrast to the accompanying decrease in osteocalcin concentrations which were observed in this group. Thus it may be hypothesised that 1,25 dihydroxy-vitamin D<sub>3</sub>, although involved in the initiation of osteocalcin release has no role to play in its



subsequent maintenance. Mongin & Sauveur (1979) also suggest that 1,25 dihydroxy-vitamin D<sub>3</sub> is probably not involved in the short term changes in calcium homeostasis occurring during the laying cycle, such as those imposed by prematurely removing the egg.

The short period of hypocalcaemic conditions imposed on the hens of Group 3 did not appear to have any effect on the rate of bone matrix formation in the laying hen. Neither did it affect the mean plasma concentration of 1,25 dihydroxy-vitamin D<sub>3</sub>. This is in contrast to the situation after 2 weeks of calcium deprivation as reported by Nys *et al* (1992) and Nys (1993).

The mineralising activity of osteoblasts has been correlated with an increase in the concentration of alkaline phosphatase (Taylor *et al*, 1965; Bloom *et al*, 1958; Chenu *et al*, 1990). In the control group (group 1) the concentration of alkaline phosphatase remained constant between 8 and 17 hours postovulation, substantiating the findings of Nys *et al* (1986). Thus both bone matrix formation and mineralisation remained constant during this time, in agreement with the model proposed by Van de Velde *et al* (1985).

According to Van de Velde *et al* (1985), a significant decrease in bone matrix formation, should have been accompanied by an increase in bone mineralisation (figure 3). However, in Group 2 (expulsed egg), the concentration of alkaline phosphatase remained stable throughout the experimental period thus implying no change in the rate of bone mineralisation. This is possibly due to the time necessary to detect the lack of an egg within the shell gland pouch and thus reverse the calcium flux and hence return calcium to the bone. Further the experiment took place during the

hours of darkness so that food derived calcium was not available for bone mineralisation.

The concentrations of alkaline phosphatase recorded in the calcium deprived group (Group 3) demonstrated no significant differences when compared to the control group. These results suggest a constant rate of bone matrix mineralisation in this group accompanying the stable rate of bone matrix formation. Again a short period of calcium deprivation appears to have had no effect on the normal skeletal processes.

The conventionally used assays for total calcium and inorganic phosphate revealed a decrease in total calcium, in the control group, between 14 and 17 hours post ovulation which is in agreement with the work of Winget & Smith (1957), Paul & Snetsinger (1969), Mueller *et al* (1973) and Nys *et al* (1986). The observed decrease in total calcium paralleled the increase, between 11 and 14 hours, in inorganic phosphate as previously described by Petersen & Parish (1939), Mongin & Sauveur (1979) and Parsons & Combs (1981), and is probably related to medullary bone resorption (Van de Velde *et al*, 1985). The role of bone related calcium in the calcification of the eggshell has been reported by various groups (Taylor & Belanger, 1969; Mongin & Sauveur, 1979; Van de Velde *et al*, 1984a; Sauveur & Mongin, 1983). It appears to be mobilised after the intestinal calcium supplies have been exhausted. Gunaratne & Boorman (1996) demonstrated that the magnitude of the increase in plasma phosphate concentration reflected the degree of skeletal mobilisation, assuming a constant rate of phosphate excretion (Prashad & Edwards, 1973). Thus it is suggested, from the data herein that the observed drop in total calcium and the accompanying increase in inorganic phosphate may reflect the

excessive demand on the bone for calcium release, during what corresponds in time to the rapid phase of eggshell mineralisation.

*Calcium and inorganic phosphate increase may point to an earlier reliance on bone*

In Group 2, where the egg had been prematurely expelled from the shell gland pouch, total calcium concentration continued to decrease throughout the experimental period following a similar pattern to the control group. However, Group 2 demonstrated significantly lower levels of inorganic phosphate from 11 hours post ovulation reflecting a decrease in bone resorption at this time (Guarantime & Boorman, 1996). Thus the drop in total calcium concentration may be the result of decreased bone resorption rather than its removal from the blood stream for transport to the shell gland pouch. This decrease in bone resorption accompanied by a decrease in the rate of bone matrix formation should, according to Van de Velde *et al* (1985) be associated with an increase in bone matrix mineralisation. However this was not evident from the results of the assay for alkaline phosphatase. Two hypotheses are offered in explanation: 1) Alkaline phosphatase is not accurately recording changes in bone matrix mineralisation in the laying hen or 2) The processes of bone matrix formation, bone resorption and bone matrix mineralisation are not accurately portrayed by the model put forward by Van de Velde *et al* (1985).

*Calcium deposited into the outer layers of the eggshell because the*

The imposition of hypocalcaemic conditions (Group 3) resulted in plasma calcium concentrations that were lower than the control group at 8 hours post-ovulation. Significantly higher inorganic phosphate concentrations were also observed in this group up to 11 hours postovulation. Candlish (1971a) found medullary bone was maximally loaded with calcium at 8 hours post ovulation, so in the light of the previous findings it is hypothesised that the lower concentrations of calcium observed in this group were

indicative of less bone mineralisation in the inter-lay period in Group 3. The observed delay in the time of the plasma calcium concentration increase may point to an earlier reliance on bone related calcium for eggshell calcification and higher level of bone resorption, as evidenced by the higher inorganic phosphate concentration in this group. Antillon *et al* (1977) also described an increase in medullary bone resorption accompanying a low calcium diet.

Magnesium ions are present within the centre of the hydroxyapatite crystals of bone and in both the mammillary cores (Itoh & Hatano, 1964) and outer palisade layer (Brooks & Hale, 1955; Itoh & Hatano, 1964) of the eggshell. The results of the control experiment demonstrate an increase in the mean plasma magnesium concentration at the end of the lay cycle. It is evident from the work of Solomon (1971) that the increased magnesium concentration in the plasma at this time ends up in the shell gland pouch because the magnesium concentration of plasma from the inferior oviducal vein, which originates at the shell gland region of the oviduct, remains constant across the lay cycle. The increase in magnesium concentration at the end of the lay cycle may correspond to the release of magnesium from the interior of the bone hydroxyapatite crystals during bone resorption. This mineral is then 'dumped' into the outer layers of the eggshell because the kidneys are unable to excrete it due to the simultaneous demand placed upon them for the removal of high concentration of phosphorous ions. Alternatively specific selection for magnesium extraction from the bone hydroxyapatite crystals may occur during the calcification of the palisade layer of the eggshell, due to the strengthening properties of magnesium in these areas (Anon, 1967). Given that hydroxyapatite is an integral component of the cuticle (Fink *et al*, 1993) it is perhaps not surprising to find



magnesium associated with these outer layers. Taylor & Kirkley (1967) suggested that the low magnesium excretion which they observed during eggshell calcification was due to increased demand for incorporation into the calcifying eggshell.

The effect of prematurely removing an egg from the shell gland pouch prevented any increase in mean plasma magnesium concentration between 11 and 14 hours post ovulation. However mean plasma calcium did rise at 17 hours post ovulation possibly as a result of magnesium secretion. This result may either reflect the lower degree of bone mobilisation in this group due to the expulsion of the egg or a lack of demand for magnesium due to the premature termination of eggshell formation.

In the calcium deprived group (Group 3) the concentration of magnesium was maintained in a similar pattern to that of the control group, although the end of lay increase in magnesium concentration was not significant in this group. The lack of any significant difference between the two groups in terms of magnesium concentration in the light of the increased rate of bone resorption previously indicated in Group 3 supports an active resorption of magnesium for incorporation into the outer layer of the eggshell.

The results contained in this chapter suggest that osteocalcin does provide a useful tool for observing the changes occurring in bone matrix formation during the eggshell calcification. In order to achieve a clear picture of all the changes occurring during eggshell calcification it however is also necessary to include markers of bone resorption. There was some doubt as to the efficacy of using alkaline phosphatase as a marker of bone matrix calcification. However, it may be possible to utilise it to isolate

changes in bone matrix mineralisation occurring with increasing age in commercial flocks.

In terms of the processes occurring within medullary bone during eggshell calcification, the pattern of change in blood related parameters in the control group and low dietary calcium group (Groups 1 & 3) follow the model proposed by Van de Velde *et al* (1985). However, in Group 2, where the calcifying egg was prematurely removed from the shell gland pouch, the anticipated decreases in both the rates of bone matrix formation and bone resorption (Figure 3) occurred earlier (Figure 12). Yet, a change in bone matrix mineralisation was not observed.

The feeding of a low calcium diet for 24 hours did not result in any changes in the rates of bone matrix formation or mineralisation during the period of eggshell calcification. Although, there was evidence to suggest lowered bone mineralisation in the inter-lay period, an earlier reliance on bone calcium and an increased rate of bone resorption.

It would appear from this study that magnesium is actively removed from the bone hydroxyapatite matrix during resorption and transported to the shell gland pouch for incorporation into the palisade layer of the eggshell.



# A COMPARISON OF EGGSHELL QUALITY AND BONE BIOLOGY BETWEEN TWO GENETICALLY DIVERGENT FLOCKS.

## 1.1 - INTRODUCTION

### **CHAPTER 3**

## A COMPARISON OF EGGSHELL QUALITY AND BONE BIOLOGY BETWEEN TWO GENETICALLY DIVERGENT FLOCKS.

### FLOCKS.

### 3 A COMPARISON OF EGGSHELL QUALITY AND BONE BIOLOGY BETWEEN TWO GENETICALLY DIVERGENT FLOCKS.

#### 3.1 INTRODUCTION

The world's current commercial laying flocks present with problems in bone integrity from hatching. Recently documented are the quality problems occurring in chicks hatching from early and late lay layer and broiler parent stock (Creel *et al*, 1998; Solomon *pers comm*). Davis Buckner *et al* (1925) expressed the opinion that the condition of the parent had a subsequent effect on the development of the embryo, however little work has been carried out relating eggshell quality, i.e. the site from which the chick first derives calcium, to chick skeletal development. Further, although the high incidence of bone breakages in mature laying stock is well documented (Gregory & Wilkins, 1989 & 1992) and corresponding deficits in eggshell quality at early and late lay have been reported (Bain, 1990; Nascimento, 1990; Nascimento *et al*, 1992, Roberts & Brackpool, 1993-4) investigations in these two related fields rarely overlap.

The calcium required for bone formation by the embryonic chick is in excess of that present in the yolk and albumen of the egg (Plimmer & Lowndes, 1924), thus 80% of the chick's hatching calcium apportionment must be gained from the eggshell (Simkiss, 1961), demonstrating the necessity for eggshell structure and composition to be as close to the genetic blueprint for perfection as possible. It has been suggested that thicker shells enable the chicks to remove a higher quotient of calcium (O'Neil & Spinks, 1953). However this phenomenon is apparently

influenced by the sex of the embryo with female chicks tending to withdraw less calcium on average from the shell than males, possibly due to their smaller skeletal systems (Kosin & Monroe, 1940). Even allowing for a high calcium content or thicker eggshell, the ultrastructural organisation of the shell must still be such that calcium is readily extractable by the developing chick. Much of the previous work carried out in this field is historical and the present study gave an opportunity to explore this area in more detail.

Previous studies relating to chick development have demonstrated that the relationships between egg weight and specific gravity are positively related to chick weight at hatching (Whiting & Pesti, 1983; Reinhart & Hurnik, 1984). Further O'Neil & Spinks (1953) correlated an increase in calcium uptake to the tibia with increasing fresh egg density. Roberts *et al* (1992) illustrated the possibility of utilising scanning electron microscopy to investigate hatched eggshell ultrastructure. These authors were able to characterise the extent of calcium withdrawal during incubation confirming previous research that suggested calcium transport from the shell to the developing chick, via the chorioallantoic membrane, takes place from day 10-12 of incubation (Tuan & Scott, 1977). Roberts *et al* (1992) were still able to identify ultrastructural variations at the mammillary level at 18 days, although no attempt was made to quantify or to relate these differences to chick development.

As described in the literature review, the eggshell is designed to facilitate gas exchange, limit mechanical damage to the embryo and act as a source of calcium during the growth phase. The hatching egg has been shown by others to illustrate the same range of structural diversity at the level of the mammillary layer,

although the incidence of specific faults is reduced (Roberts *et al*, 1992). With reference to the definitions of structural variations within the mammillary layer of the table egg (1.3.1), these also hold true for the hatched egg, i.e. faults such as Type A and B bodies, cubics, aragonite, depression and erosion, all of which reduce the bonding between shell and membrane fibres can be considered detrimental from the point of view of calcium uptake. Confluence on the other hand is likely to impede gaseous exchange because of the fusion of adjacent mammillary bodies effectively blocking pore sites. Such low porosity has been demonstrated to not only limit the available oxygen within the egg (Tullet & Deeming, 1982) but also result in a decrease in embryonic metabolism and growth rate (Burton & Tullet, 1983). The structural modifications early and late fusion will alter the effective thickness of the eggshell and hence the ease with which the embryo can pip the same. Given the utilisation of the mammillary caps during calcium uptake by the developing chick it is not possible to comment on either cap quality or the presence of changed membrane in the hatched eggshell.

Although the predominant mineral of the eggshell is calcium, magnesium is present in the mammillary cones (Solomon, 1986) demonstrating a potential requirement during chick development. Phosphorus is also present within the eggshell, and is utilised in bone formation, although a finite balance is necessary as phosphorus is an acknowledged crystal poison which in excess is detrimental to eggshell quality (Simkiss, 1969; Sauveur & Mongin, 1983).

During the 10 day developmental period, chick bone must form to the point at which the hatched chick is able to fend for itself, such development is termed precocial. The complete skeletal system is

derived through two bone growth processes; viz. intramembranous and endochondrial ossification (Gay, 1988). For the purposes of this chapter histomorphometry will be discussed with reference to the long bones as these are the most important bones when considering eggshell calcification, providing not only support but also medullary bone, a labile calcium source. In the long bones of the developing embryo the processes of intramembranous and endochondrial ossification occur simultaneously. Endochondrial ossification occurs at the epiphyses of the bone where five zones of development are apparent. At the top of the ossification centre is the resting zone which consists of hyaline cartilage, this zone merges with the proliferative zone where the chondrocytes (the main cellular constituents of cartilage) divide rapidly to form parallel rows of cells. In the hypertrophic zone the chondrocytes increase in size through the accumulation of glycogen in the cytoplasm. The surrounding mucoid matrix of the cells is much reduced in this zone. Within the calcified cartilage zone the chondrocytes die, as they are isolated from their source of nutrition, subsequently their matrix becomes calcified by the deposition of hydroxyapatite by the osteoblasts. In the zone of ossification the osteoblasts form layers over the surface of the former cartilage matrix and continue to form and calcify the bone matrix.



At hatch the chick has a fully functional skeletal system. From this stage until immediately preceding lay, the skeleton serves a purely supportive role. To this end it maintains the ability to grow through endochondral ossification at the growth plates.

Approximately 14-16 days prior to the onset of lay, corresponding to ovarian follicle maturation (Dacke *et al*, 1993), medullary bone formation commences (Hurwitz, 1964)(Chapter 4). Medullary bone with its good vascular supply (Bloom *et al*, 1958) and loose fibre structure provides a readily mobilisable source of calcium with remodelling occurring 10-15 times faster than in structural bone (Murray & Bloom, 1948; Hurwitz, 1965). Hurwitz (1964) demonstrated 70% turnover of the calcium within medullary bone in 12 days. The presence of medullary bone increases bone weight by 20% with the greatest mass being achieved immediately prior to shell calcification (Candlish, 1971a).

With the onset of lay the skeletal system of the female bird adopts a new role, that of mineral provision for the calcifying eggshell. Eggshell calcification occurs between 3 and 23 hours post ovulation during which time both medullary bone matrix formation and resorption are occurring maximally (Van de Velde *et al*, 1984a). The experiment carried out in chapter 2 confirmed the use of osteocalcin as a marker of medullary bone matrix formation during the period of eggshell calcification. This marker demonstrated a constant rate of medullary bone matrix formation in mid lay commercial laying hens, during the period 8-17 hours post ovulation, accompanied by no change in the concentration of its stimulant 1,25 dihydroxy-vitamin D<sub>3</sub>. A decrease in the concentration of plasma total calcium was recorded over the experimental period demonstrating the demand for calcium by the calcifying eggshell. The increasing concentration of inorganic



phosphate during calcification served as a marker for bone resorption. Inorganic phosphorus is released by the bone matrix on resorption and subsequently accumulates within the blood stream due to the inability of the kidneys to process the increased levels (Prashad & Edwards, 1973). The newly formed osteoid matrix is mineralised in the period between successive eggs (Van de Velde *et al*, 1985). Chapter 2 demonstrated the low levels of bone matrix mineralisation during eggshell calcification as endorsed by the steady levels of alkaline phosphatase. However attention was drawn to the inability of this marker to detect subtle changes in bone remodelling after premature egg expulsion. Finally, it was concluded in the previous study that active selection was occurring for magnesium incorporation into the outer layers of the eggshell.

Having established the changes in blood parameters occurring during a single lay cycle (Chapter 2) the next step was to establish how these parameters varied with bird age.

Bone matrix mineralisation occurs in the inter-egg period. In the currently available commercial hybrids this time is reduced to a period of approximately 6 hours between successive periods of eggshell calcification. During the laying life of the hen structural bone is constantly decreasing in volume, possibly due to the replenishing of medullary reserves (Taylor & Dacke, 1984; Thorp *et al*, 1993; Knott *et al*, 1995), to such an extent that late lay bone breakages are common. In the trabecular bone of mature commercial laying hens it has been noted that there is a lack of osteoid and functional osteoblasts (Whitehead & Wilson, 1992). In less selected lines the period is much extended by the laying of eggs in well defined clutches with 2-4 day breaks during which time bone matrix may be mineralised. Rennie *et al* (1997)

compared a strain of bird, the J.Line, which has been unselected for approximately 40 years, with a commercial hybrid. According to these authors the relatively poor egg production of the J.Line may reduce their tendency for continuous structural bone loss during lay, thereby enabling them to regenerate structural bone during the periods when they are out of lay. The authors concluded that the development of osteoporosis in commercial flocks was attributable to the length of the period of continuous reproduction, rather than to the number of eggs produced *per se* (Whitehead Wilson, 1992; Rennie *et al*, 1997). However, Rennie *et al* (1997) did not state the time of plasma sampling, which has since been demonstrated to be of the utmost importance (Figure 3). Further, while production was measured, eggshell quality was not considered.

Poor eggshell quality at the ultrastructural level is routinely found in eggs which have passed normal eggshell quality controls. The examination of the 12 ultrastructural variations of the mammillary layer (Reid, 1984) (1.3) reveals differences with age and strain (1.4 & 1.5). As egg size increases with maturity in the laying hen differences are evident in production, eggshell quality and eggshell strength, which Peterson (1965) associated with a decrease in available mineral within the skeleton. Bell & Siller (1962) hypothesised that the osteoporotic bird lacked a mechanism to regulate egg production when bone stocks were depleted. It can be hypothesised that the observed decrease in positive eggshell characteristics (Bain, 1990 & 1992) at late lay is related to the poor bone structure observed at this time.

The first aim of this chapter was to identify any ultrastructural variation between a small subsample of successfully hatched eggshells from a highly selected commercial hybrid line and the

relatively unselected brown leghorn (J.Line). Quantification was based on a modified scoring system taking into account the role of specific inclusions on shell structure and function. The relationship between eggshell quality and bone development was then investigated in the two divergent strains.

The second aim of this chapter was to carry out a study of eggshell quality, bone histomorphology and blood parameters at early (25-30 weeks), mid (45 weeks) and late (67 weeks) lay, in 2 genetically divergent lines to establish if selection for increased egg numbers has influenced the inter-relationship between these parameters. The hypothesis that the deficits in eggshell quality at early and late lay are attributable to bone histomorphometry was investigated.

## **3.2 MATERIALS & METHODS**

### **3.2.1 EXPERIMENT 1**

**i) General experimentation** 30 eggs from each of a modern hybrid (strain 1) and J.Line (strain 2) mid lay parent stock were hatched in individual pens, allowing hatched eggshells to be related to individual chicks. 15 female chicks from each strain were sacrificed immediately and their left femur dissected for mineral assessment. Corresponding eggshells from the sacrificed chicks were collected for ultrastructural and mineral assessment.

**ii) Bone Mineral Analysis.** Each femur was fat extracted with petroleum spirit (bp 40-60°C) for 70 minutes and rinsed for 30 minutes. The samples were then dried to constant weight in an oven at 100°C for approximately 4 hours. The weight of the dry fat free bone was recorded prior to ashing in a muffle furnace at 550°C overnight. The resultant ash was then cooled and reweighed to allow calculation of % bone ash of dry fat free weight.

The ash was extracted by the addition of 6N Hydrochloric acid to a ratio of 1:5 of the final volume and evaporated to dryness on a hot plate. The precipitate was re-dissolved with heating in the same volume of 6N Hydrochloric acid before being made up with distilled water in a volumetric flask. Final volume depends on bone size in this case 25ml. This solution was used in the determination of phosphorus, calcium and magnesium.

Phosphorus was determined colorimetrically using the TRAACS 800 automated chemistry system which automatically calculates

phosphorus as ppm. The process involved the extracted bone solution being mixed with Molydovanadate Reagent which resulted in a yellow solution which can be measured at 420nm.

Both calcium and magnesium were determined by Atomic Spectrophotometry at wavelengths of 422.7nm and 285.2nm, respectively. In the case of calcium a Lanthanum salt was added to eliminate the interference of other elements.

### **iii) Ultrastructural assessment of eggshell quality.**

#### **a) Ultrastructural assessment by weighted score.**

Ultrastructural assessment of table eggshell quality consists of an incidence appraisal of each of twelve structural variants found in the mammillary layer of the hens eggshell (Figure 2) (Reid, 1984; Watt, 1985, 1989; Bain, 1990; Solomon, 1991; Fraser, 1996). Each variant is assigned a score depending on its incidence in the eggshell. The score assigned to each individual variable is weighted in terms of its perceived importance on shell performance (Figure 13). At the end of the analysis the total score is calculated.

The scoring system in this experiment was modified to take account of the fact that during calcium uptake by the developing chick the basal layers of the shell undergo degradation thus, mammillary caps disappear and changed membrane is rarely observed (Figure 14). The weighted values for calcium withdrawal relate to the degree of erosion from the mammillary caps. The occurrence of early fusion has been heavily weighted against with reference to the fact that it increased the effective thickness of the shell and so may interfere with pipping.



CONFLUENCE	NONE/ISOL 1	ISOL/MOD 3	MOD/EXT 7		
CAPS	GOOD 1	AVERAGE 3	BELOW AVERAGE 5	POOR 7	
FUSION	EARLY 1	MAINLY EARLY 3	50:50 5	MAINLY LATE 7	LATE 10
ALIGNMENT	RANDOM 1	MOD. ORGAN 3	FISSURED 5		
TYPE B	NONE/ISOL 1	ISOL/MOD 3	MOD/EXT 7		
DEPRESSION	NONE 1	LIMITED 2	MODERATE 4	DETRIMENTAL 6	
EROSION	NONE 1	LIMITED 3	MODERATE 5	DETRIMENTAL 8	
ARAGONITE	NONE/ISOL 1	ISOL/MOD 3	MOD/EXT 7		
TYPE A	NONE/ISOL 1	ISOL/MOD 3	MOD/EXT 7		
CUBIC	NONE/ISOL 1	ISOL/MOD 3	MOD/EXT 7		
CUFFING	NONE/ISOL 7	ISOL/MOD 3	MOD/EXT 1		
CHANGED MEMBRANE	NONE/ISOL 1	ISOL/MOD 4	MOD/EXT 7		

Figure 13: Shell Quality Weighted Score System for table eggs.

CONFLUENCE	NONE/ISOL 1	ISOL/MOD 3	MOD/EXT 7		
FUSION	EARLY 10	MAINLY EARLY 8	50:50 3	MAINLY LATE 4	LATE 7
ALIGNMENT	RANDOM 1	MOD. ORGAN 3	FISSURED 5		
TYPE B	NONE/ISOL 1	ISOL/MOD 3	MOD/EXT 7		
DEPRESSION	NONE 1	LIMITED 2	MODERATE 4	DETRIMENTAL 6	
EROSION	NONE 1	LIMITED 3	MODERATE 5	DETRIMENTAL 8	
ARAGONITE	NONE/ISOL 1	ISOL/MOD 3	MOD/EXT 7		
TYPE A	NONE/ISOL 1	ISOL/MOD 3	MOD/EXT 7		
CUBIC	NONE/ISOL 1	ISOL/MOD 3	MOD/EXT 7		
CUFFING	NONE/ISOL 1	ISOL/MOD 3	MOD/EXT 7		
CALCIUM WITHDRAWAL	GOOD 1	AVERAGE 3	BELOW AVERAGE 5	POOR 7	

Figure 14: Shell Quality Weighted Score System modified for hatched eggs.



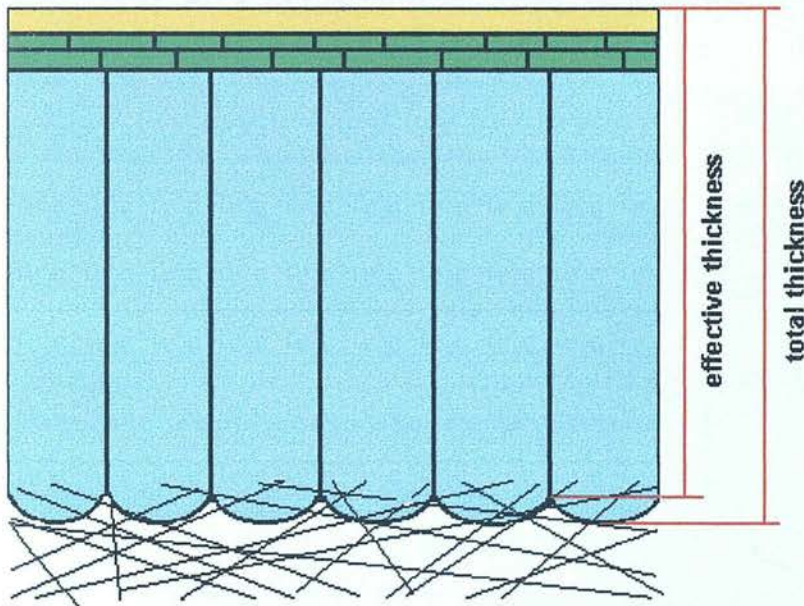
An appraisal of eggshell ultrastructure is only conceivable using scanning electron microscopy and thus a specialised preparation procedure was followed.

A  $1\text{cm}^2$  section was removed from the midline of each eggshell using a diamond tipped, circular saw mounted on a dentist's drill (NM 3000, Novag, Switzerland). The sections were soaked in distilled water for 2 minutes to facilitate the manual removal of the shell membranes. In hatched eggshells both membranes are readily removable (cf. 3.2.2 iii). The sections were then oven dried at  $60^\circ\text{C}$  for 20 minutes on a glass petri dish. The sections were then mounted, cuticle down, on aluminium stubs with conductive silver paint before being oven dried at  $60^\circ\text{C}$  for 30 minutes. Contrast was achieved by the coating of the prepared sections with gold/palladium (Emscope Sputter Coater SC 500, Ashford, Kent, England).

The incidence of the twelve structural variants was assessed using a Phillips 501B scanning electron microscope (Kv 14, spot size 250) (Philips, Holland) and total ultrastructural score was calculated.

**b) Ultrastructural assessment by thickness profiles.** A  $1.5 \times 0.5\text{cm}$  section was removed from the equator of each egg using a diamond tipped, circular saw mounted on a dentists drill (NM 3000, Novag, Switzerland). Each section was then manually snapped in two before being mounted edge on in a grooved aluminium stub, using conductive silver paint. The stubs were coated with gold palladium before being viewed using the Philips 501B scanning electron microscope at 15Kv, working distance 13 and magnification  $\times 160$ . Sections were tilted so that neither the upper nor lower surface was in view. Several

measurements were then taken to the nearest 1/100mm of the remaining thickness (Figure 15) (the distance from the cuticular layer to the remaining surface of the utilised mamillary body) and the remaining mamillary layer thickness (the distance from the point of fusion of the palisade columns to remaining surface of the mamillary body). The effective thickness (the distance from the cuticular layer to the point of fusion of the palisade columns) was then calculated by subtraction (Bain, 1990).



**Figure 15:** A schematic representation of the eggshell demonstrating total and effective thickness. (For hatching eggs total thickness = remaining thickness)

**v) Eggshell mineral analysis.** Approximately  $1\text{cm}^2$  sections were taken from the equator of each eggshell described in 3.2.1 iii & iv. The sections were soaked in distilled water for 2 minutes to allow the manual removal of the shell membranes. Mineral analysis took place according to the procedures described in 3.2.1ii, with the exception of the fat extraction stage.

**vi) Data analysis.** Data were analysed using the Minitab statistical package (Release 11; Minitab Inc, Pasadena, USA). Data were inserted into a general linear model to assess the nature of the relationships between the strains. All significant data were also graphed and error bars calculated.

### **3.2.2 EXPERIMENT 2**

**i) Production variables.** 45 modern hybrid (strain 1) and 45 J.Line (strain 2) chicks were hatched and housed in a brooder until four weeks of age before being transferred to individual bird cages. Standard management techniques were applied in all stages of development (Appendix 3, Roslin Institute *pers comm*). Care was taken to avoid cage position bias within and between the groups of birds, thus equal numbers of birds were placed randomly in both tiers of the system. A full dietary analysis can be seen in Appendices 1 and 2. From 5% lay (approximately 18 weeks in strain 1 and 23 weeks in strain 2) production records were kept. These detailed the number of eggs laid per hen per week and the % seconds per hen per week (those eggs which would be routinely graded out on farm) (Appendix 4). Egg weight was recorded during the experimental periods (p/hen/day).

**ii) General experimentation.** At 25 (30 weeks in strain 2), 45 and 67 weeks of age, 15 birds were selected at random from each group and treated as follows: 14 consecutive eggs per bird (where possible) were collected, pooled on a group basis and subjected to ultrastructural assessment; the eggs laid on the fifteenth day were collected to allow the assessment of traditional quality parameters and mineral composition. The 15 birds were then blood sampled prior to sacrifice for bone biology assessment. Blood sampling and sacrifice took place at approximately peak eggshell mineralisation (10 hours post-oviposition) which was determined by manual recording of the previous egg laid. Differences in the timing of the first sampling period were due to differences in the time of coming into lay.



### **iii) Ultrastructural assessment of eggshell quality.**

#### **a) Ultrastructural assessment by weighted score.**

Eggs

were emptied by excision of the pointed pole. As described in 3.2.1 iii, a  $1\text{cm}^2$  section from the midline of each eggshell was soaked in distilled water for 2 minutes to facilitate the manual removal of the inner shell membranes. The sections were then oven dried at  $60^\circ\text{C}$  for 20 minutes on a glass petri dish. The outer membrane fibres were removed by plasma etching (Reid, 1983)(Nanotech 100 Plasma Chemistry Unit) for 4 hours in an atmosphere of oxygen (133.3 Pascals) volatilised by a radio frequency of 100 ohms. The remaining ash was blown away with a jet pressure duster. Sections were mounted and coated as described in 3.2.1 iii. Sputter Coater SC 500, Ashford, Kent, England). The incidence of the twelve structural variants was assessed in the first instance using the scoring system described in Figure 14, utilising a Tesler BS343. The presence of changed membrane was confirmed using an EDAX analyser (PV9800, Edax Inc, USA) (Watt, 1985, 1989). The individual scores for each egg were summed to reach a value for total ultrastructural score.

#### **b) Thickness profiles.**

The eggs utilised in sections 3.2.2 iv and v were emptied by excision of the pointed pole and utilised in a study of thickness profiles (3.2.1 iv). In this instance measurements were taken of the total thickness (Figure 15) (the distance from the cuticular layer to the tip of the mammillary body) and the mammillary layer thickness. The effective thickness was then calculated by subtraction (Bain, 1990).

#### iv) Traditional methods of assessing eggshell quality.

Traditional methods of assessing eggshell quality involve consideration of the egg as a whole and includes values for weight, shape index, deformation, breaking strength and stiffness. Only those eggs which were intact and devoid of any surface defects were considered for assessment.

All eggs were weighed to the nearest 0.1g (Oertling TP30). The length and breadth were subsequently taken to the nearest 0.1mm using hand callipers. From these measures the shape index was derived as follows:

$$\text{Shape index} = \frac{\text{length}}{\text{breadth}}$$

Deformation was then assessed using the Mauris non-destructive deformation tester (N.V. Hollantlenn 18, Utrecht, Netherlands). The mean deformation was derived from three separate measurements taken around the equator of each egg.

Quasistatic compression tests were subsequently carried out by compressing each egg at the equator using a J.J. Lloyd screw driven testing machine fitted with a 100N load cell. The compression speed was standardised at 5cm/minute. Displacement was measured throughout the test using a transducer coupled to an XY chart recorder. The force ( $y=F$ ) and deformation ( $x=d$ ) were measured throughout the test with slope equal to shell stiffness (Bain, 1990). The maximum force exerted on the shell prior to an audible crack and a sharp drop in the force/displacement relationship equalled shell breaking strength.



### **v) Assessment of the material properties of the eggshell.**

The material properties of the eggshell (elastic modulus and fracture toughness) allow direct comparisons to be made between samples by taking into account differences in shape, curvature and thickness (Bain, 1990).

**a) Elastic modulus.** The elastic modulus describes the forces acting in the eggshell under load in relation to the stiffness of the shell. It was calculated using the method as described by Bain (1990):

$$E. \text{ shell} = \frac{C F R}{dt^2}$$

where

E. shell = elastic modulus

t = effective thickness

C = compliance or correction for egg shape

R = radius of curvature = (breadth/2)

F/d = (force/displacement)=stiffness

and

$$C = C_{\text{sphere}} \times A$$

where

$$C_{\text{sphere}} = \frac{0.408 + 3.026t^2}{b}$$

and

$$A = \frac{C_{\text{eggshape}}}{0.444}$$

$$= \frac{-0.666 + (1.866 \times SI) - (0.907 SI^2) + (0.153 SI^3)}{0.444}$$

where

b = breadth, SI = shape index

**b) Fracture toughness.** The fracture toughness describes the relationship between the force necessary to produce eggshell failure and the size of any defects in the shell which may contribute to its failure. Fracture toughness was also calculated using the method as described by Bain (1990):

$$K_c = K_{nd}(F/t^{3/2})$$

where

$K_c$  = fracture toughness

$F$  = force at fracture = breaking strength

$K_{nd} = 0.777(2.388 + 29.934\sigma)^{1/2}$

$t$  = effective thickness

$\sigma$  = critical crack length / radius of curvature

Note: critical crack length was assumed to be 6mm (after Bain, 1990).

**vi) Egg shell mineral analysis.** Approximately  $1\text{cm}^2$  sections were taken from the equator of each eggshell described in sections 3.2.2 iv and v. Sections were prepared to the post plasma etching stage (3.2.2iia) before mineral analysis took place according to the procedures described in sections 3.2.1 ii & v, with the exception of the fat extraction stage.

**vii) Blood samples.** A single time point was selected within the balanced period of eggshell calcification (Figure 3) at approximately 10 hours post ovulation. Blood samples of approximately 5ml were taken from the wing vein, in heparinised syringes, and assayed immediately for ionised calcium and pH before being spun down (3000G, 5 minutes) and plasma stored at  $-20^\circ\text{C}$  until needed. 2ml aliquots of plasma for osteocalcin

analysis were stored at  $-70^{\circ}\text{C}$ , in dry ice for transportation purposes.

Due to the nature of this experiment it was considered unlikely that any changes in 1,25 dihydroxy-vitamin  $\text{D}_3$  would be evident as both the results of chapter 2 and the studies of previous authors have led to the conclusion that it is unlikely to be involved in the short term changes occurring during the lay cycle (Mongin & Sauveur, 1979). Technical considerations made it impossible to carry out the assay for inorganic phosphate in this experiment, thus a measure of total phosphorus (Wako kit, Alpha Laboratories, Hampshire) was substituted which evaluates phosphorus from both dietary and bone sources. Due to the consistency of dietary conditions at any one time across this experiment any changes detected at this time can be assumed to be due to changes in bone remodelling. The kit for total phosphorus was modified for use with avian plasma (two-fold dilution, Rennie *et al*, 1997) and for measurement with an automatic plate reader (Titertek Twin-Reader Plus, ICN) (Whitehead *pers comm*).

In chapter 2 a measure of total calcium was utilised and while this provided a valuable insight into the plasma calcium concentration during the period of eggshell calcification, it was decided in this experiment to utilise an assay for ionised calcium and pH which is similarly affected by reproductive state, photoperiod and feed (Parsons & Combs, 1981). Assays were also carried out on alkaline phosphatase, and magnesium. For further details see 2.2.

### viii) Bone histomorphometry.

#### a) Bone Volume Assessment.

Approximately 1cm samples were taken from the left proximal tarsus metatarsus (PTM) and decalcified. Sections were then stained to allow assessment of bone volume.

Samples were primarily fixed in 10% buffered neutral formalin for 7 days. Decalcification then took place in Gooding Stewarts Fluid consisting of 1 part formaldehyde, 1 part formic acid, to 7 parts distilled water, after which samples were washed in tap water overnight before being returned to buffered neutral formalin. Decalcification was determined through X-ray analysis (Faxitron 804, Hewlett Packard, USA). They were then processed through ascending alcohol concentrations and CNP 30 to paraffin wax. 4 $\mu$ m interrupted serial sections, 1000 $\mu$ m apart, were cut using a microtome (Lietz Rotary) and stained with toluidine blue.

Samples for bone volume analysis were deparaffinised and hydrated by a process of CNP 30 and descending alcohol concentrations, stained in 1% toluidine blue in 5% EDTA for 5 minutes, before being dehydrated cleared and mounted.

The relative amounts of both medullary and trabecular bone were measured by semi-automated image analysis (Fleming *et al*, 1994) using an Apple MacIntosh-based system running NIH Image 1.47 (public domain software). This allowed the measurement of 10 fields per section, on three sections per bone. Mean volumes were then calculated.

**b) Bone Density Assessment.** The left humerus was dissected and radiographed (Faxitron 804, Hewlett Packard, USA). The films were digitised using a monochrome video camera (Panasonic WVBL600) and bone density (mm Al equivalent) was measured using NIH Image 1.47 (Fleming *et al*, 1994). Both bone and marrow width were also analysed using this system. This allowed the calculation of the variable K, as described by Currey & Alexander (1985).

$$K = \frac{m}{b}$$

where m = marrow width

and b = bone width

**c) Bone Mineral Analysis.** Approximately 0.5 cm bone samples were taken from the central shaft of the left femur. Each sample was subjected to analysis for calcium, phosphorus and magnesium as described in section 3.2.1 ii.

**ix) Data analysis.** Data were analysed using the Minitab statistical package (Release 11; Minitab Inc, Pasadena, USA). Data were inserted into a general linear model to assess the nature of strain|lay interactions. A post-hoc t-test was carried out on all significant data, using pooled standard error values. All significant data were also graphed and standard deviations calculated.

### **3.3 RESULTS**

#### **3.3.1 EXPERIMENT 1**

The GLM carried out to determine if there are differences between Strain 1 and 2 in terms of chick bone mineral composition and the ultrastructural quality and mineral composition of hatched eggshells are presented in Tables 3-5, accompanied by the standard deviation of the mean.

i) **Bone mineral assessment.** There was no significant difference in bone mineral composition between the two strains (Table 3).

ii) **Ultrastructural eggshell quality.** Total ultrastructural score varied significantly between the two strains, with strain 2 having a higher score and thus poorer quality eggshells (Table 4; Figure 16). This difference was largely due to a higher incidence of early fusion (Figure 17) and cuffing (Figure 18) in the strain 2 hatched eggs. The hatched eggshells of both strains were of similar thickness.

iii) **Eggshell mineral analysis.** The hatched eggshells of strain 1 demonstrated a significantly higher ash and phosphorus content than those of strain 2 although within strain variation was also high. There was no significant difference in the proportions of calcium between the two strains (Table 5; Figure 19). Strain 2 displayed significantly higher magnesium concentrations within the hatched eggshell than strain 1 (Figure 20).





VARIABLE	% ASH (dry fat free)		% CALCIUM		% PHOSPHORUS		% MAGNESIUM	
	1	2	1	2	1	2	1	2
STRAIN								
MEAN (st.dev)	21.77 (2.22)	21.08 (2.52)	8.62 (1.18)	8.87 (1.05)	4.18 (0.59)	4.45 (0.46)	0.24 (0.08)	0.23 (0.07)
Strain	ns		ns		ns		ns	

**Table 3:** Influence of strain on bone mineral parameters in the newly hatched chick.

(st. dev) = standard deviation of the mean; \*\*  $p < 0.01$ ; \*  $p < 0.05$ ; ns not statistically significant

VARIABLE	CONFLUENCE		FUSION		CUFFING		ALIGNMENT		TYPE B		DEPRESSION		EROSION		CUBIC		ARAGONITE	
STRAIN	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
mean	1.80	1.80	4.40	7.27	1.00	2.60	2.93	3.13	2.20	2.67	1.53	1.40	2.07	1.67	1.53	1.80	1.00	1.00
(st.dev)	(1.01)	(1.66)	(1.58)	(1.06)	(2.03)	(0.00)	(1.33)	(0.52)	(1.66)	(0.72)	(0.83)	(0.63)	(1.49)	(0.98)	(1.60)	(1.60)	(0.00)	(0.00)
Strain	ns		**		**		ns		ns		ns		ns		ns		ns	

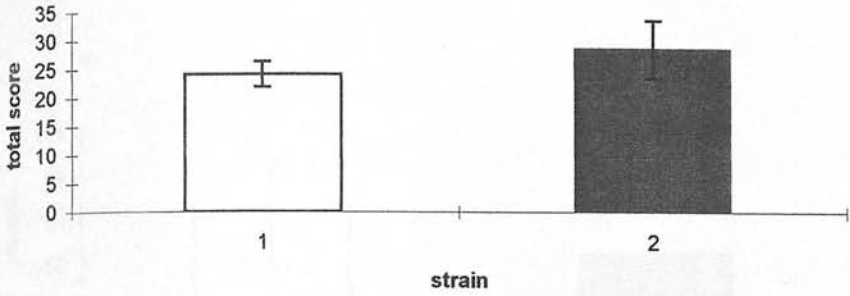
VARIABLE	CALCIUM EROSION		TYPE A		TOTAL		REMAINING THICKNESS (mm)		MAMMARY THICKNESS (mm)		EFFECTIVE THICKNESS (mm)	
STRAIN	1	2	1	2	1	2	1	2	1	2	1	2
mean	4.40	4.07	1.13	1.13	24.00	28.53	0.30	0.25	0.04	0.03	0.26	0.21
(st.dev)	(1.92)	(2.12)	(0.52)	(0.52)	(2.23)	(5.07)	(0.02)	(0.02)	(0.01)	(0.01)	(0.02)	(0.02)
Strain	ns		ns		**		ns		ns		ns	

**Table 4:** Influence of strain on ultrastructural eggshell quality variables in the hatched egg. (st. dev) = standard deviation of the mean; \*\*  $p < 0.01$ ; \*  $p < 0.05$ ; ns not statistically significant

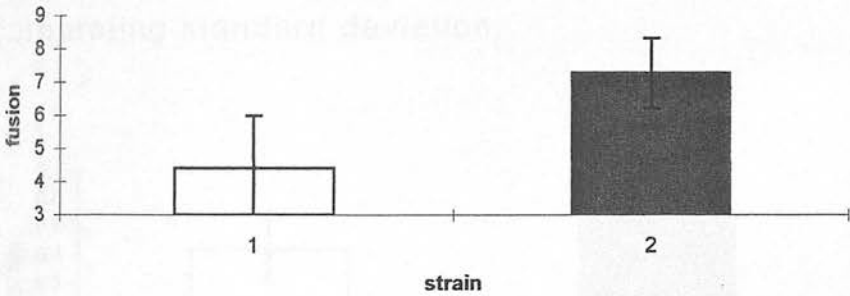
VARIABLE	% ASH (dry fat free)		% CALCIUM		% PHOSPHORUS		% MAGNESIUM	
	1	2	1	2	1	2	1	2
STRAIN								
MEAN (st.dev)	94.52 (5.96)	89.98 (1.36)	33.05 (2.98)	31.19 (4.19)	0.066 (0.59)	0.033 (0.46)	0.42 (0.08)	0.53 (0.11)
Strain	**		ns		**		**	

**Table 5:** Influence of strain on eggshell mineral in the hatched egg.

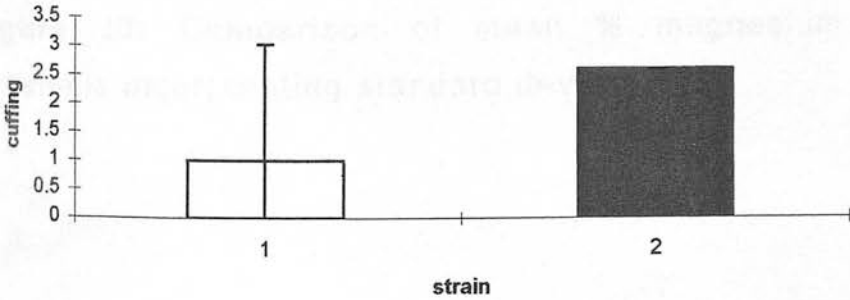
(st.dev) = standard deviation of the mean; \*\*  $p < 0.01$ ; \*  $p < 0.05$ ; ns not statistically significant.



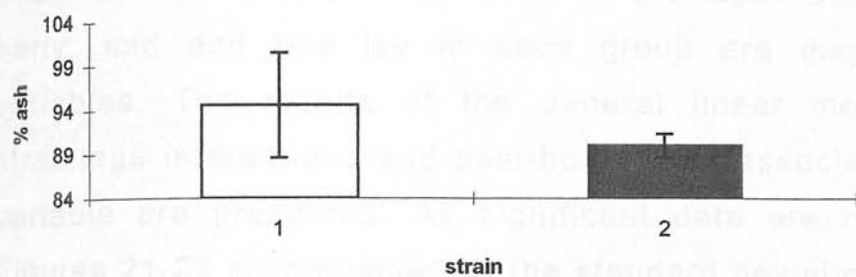
**Figure 16:** Comparison of mean total ultrastructural score in hatched eggshells incorporating standard deviation.



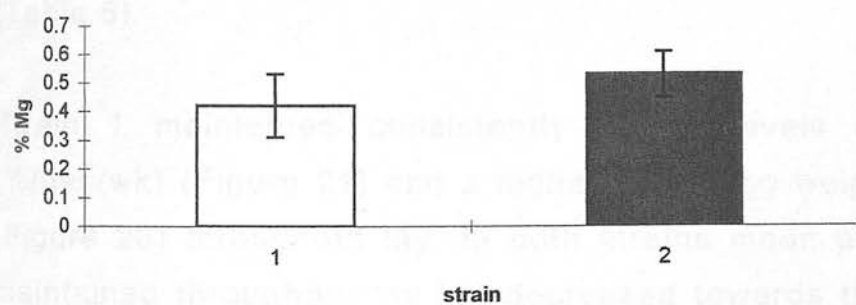
**Figure 17:** Comparison of mean total fusion scores in hatched eggshells incorporating standard deviation.



**Figure 18:** Comparison of mean total cuffing scores in hatched eggshells incorporating standard deviation (where no bar shown standard deviation = 0)



**Figure 19:** Comparison of mean % ash in hatched eggshells incorporating standard deviation.



**Figure 20:** Comparison of mean % magnesium in hatched eggshells incorporating standard deviation.



### 3.3.2 EXPERIMENT 2

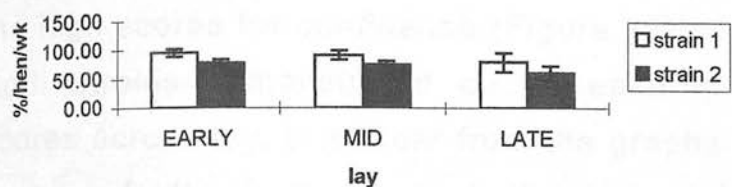
The mean and standard deviation of the mean corresponding to early, mid and late lay in each group are displayed for all variables. The results of the general linear model (glm) for strain|age interactions and post-hoc t-tests associated with each variable are presented. All significant data are represented in Figures 21-23 accompanied by the standard deviation of the mean values.

**i) Production variables.** In order to assess differences in production, seconds and egg weight data over lay, the mean of three weeks of data prior to experimentation was calculated (Table 6).

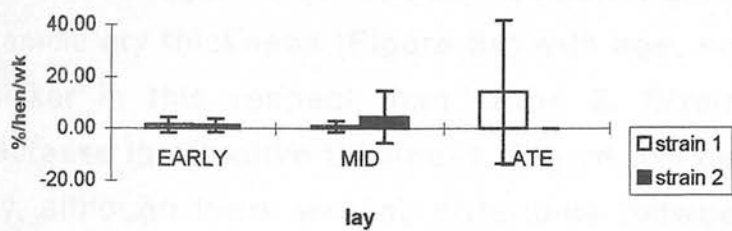
Strain 1 maintained consistently higher levels of production (%/hen/wk) (Figure 21) and a higher mean egg weight (g/hen/wk) (Figure 23) throughout lay. In both strains mean production was maintained throughout lay but decreased towards the end of lay, accompanied by an increase in inter-bird variation. Egg weight increased in both strains between early and mid lay and then remained stable to late lay. The % seconds (Figure 22) in strain 1 increased rapidly at late lay, although variability was high.

VARIABLE	PRODUCTION (%/hen/wk)		EGG WEIGHT (g/hen/day)		SECONDS (%/hen/wk)	
	1	2	1	2	1	2
STRAIN						
EARLY (st.dev)	95.6 <sup>a</sup> (6.58)	77.78 <sup>b</sup> (6.70)	61.24 <sup>a</sup> (3.54)	47.17 <sup>b</sup> (2.82)	1.46 <sup>a</sup> (3.00)	1.24 <sup>a</sup> (2.57)
MID (st.dev)	91.21 <sup>a</sup> (7.74)	74.67 <sup>b</sup> (7.05)	70.44 <sup>b</sup> (3.93)	56.37 <sup>c</sup> (7.56)	0.88 <sup>ab</sup> (2.18)	4.51 <sup>a</sup> (10.08)
LATE (st.dev)	79.59 <sup>b</sup> (16.43)	61.00 <sup>c</sup> (11.82)	67.89 <sup>b</sup> (13.03)	59.32 <sup>c</sup> (5.84)	14.21 <sup>b</sup> (27.50)	0.00 <sup>a</sup> (0.00)
Strain	**	**	**	**	ns	ns
Age	**	**	**	**	ns	ns
Strain Age	ns	ns	ns	ns	*	*

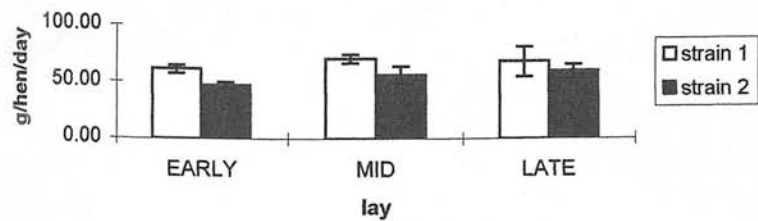
**Table 6:** Influence of strain and age on production variables in the laying hen. Mean values are given. (st.dev) = standard deviation of the mean; a-c = means with same superscript do not differ significantly ( $p < 0.05$ ); \*\*  $p < 0.01$ ; \*  $p < 0.05$ ; ns not statistically significant.



**Figure 21:** Mean production over lay incorporating standard deviation.



**Figure 22:** Mean % seconds over lay incorporating standard deviation.



**Figure 23:** Mean egg weight over lay incorporating standard deviation.

**ii) Ultrastructural measures.** Strain 1 displayed a higher ultrastructural score (Figure 24) at early lay than strain 2 due to the high scores for confluence (Figure 25) and cubics (Figure 30). Both strains demonstrated an increase in total ultrastructural scores across lay. It is clear from the graphs that the incidence of specific faults fluctuate in both strains throughout the study period. Differences between the two strains with reference to individual variables are highlighted in Table 7 and Figures 25-32.

Total thickness (Figure 33) remained constant across lay in strain 1 whilst strain 2 displayed a substantial decrease at late lay leading to a significant difference between the two strains at this time. Although both strains demonstrated a consistency in mamillary thickness (Figure 34) with age, strain 1 is consistently thicker in this respect than strain 2. Strain 2 demonstrated a decrease in effective thickness (Figure 35) between early and mid lay, although there was no difference between the two strains in terms of this measure.

VARIABLE	CONFLUENCE		FUSION		CUFFING		ALIGNMENT		TYPE B		DEPRESSION		EROSION		CUBIC		ARAGONITE	
	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
STRAIN																		
EARLY (0.00)	3.15 <sup>a</sup> (0.38)	1.89 <sup>c</sup> (0.00)	7.62 <sup>a</sup> (1.04)	6.78 <sup>a</sup> (1.30)	7.00 (0.00)	7.00 (0.00)	3.00 <sup>a</sup> (0.00)	3.00 <sup>a</sup> (0.00)	3.15 <sup>ac</sup> (0.38)	3.00 <sup>c</sup> (0.00)	1.00 (0.00)	1.00 (0.00)	1.15 <sup>a</sup> (0.55)	1.00 <sup>ac</sup> (0.00)	2.46 <sup>a</sup> (0.88)	1.00 <sup>b</sup> (0.00)	1.54 (1.13)	1.00 (0.00)
MID (st.dev)	1.92 <sup>b</sup> (0.76)	3.2 <sup>d</sup> (0.84)	7.54 <sup>bd</sup> (0.88)	6.6 <sup>ad</sup> (1.14)	7.00 (0.00)	7.00 (0.00)	3.15 <sup>b</sup> (0.55)	3.00 <sup>a</sup> (0.55)	3.39 <sup>ab</sup> (0.51)	3.00 <sup>ac</sup> (0.00)	1.00 (0.00)	1.00 (0.00)	1.39 <sup>a</sup> (0.51)	1.00 <sup>c</sup> (0.00)	1.23 <sup>b</sup> (0.60)	1.00 <sup>b</sup> (0.00)	1.08 (0.28)	1.00 (0.00)
LATE (st.dev)	1.00 <sup>c</sup> (0.00)	3.33 <sup>d</sup> (1.03)	4.71 <sup>c</sup> (0.95)	4.00 <sup>bc</sup> (0.63)	7.00 (0.00)	7.00 (0.00)	3.14 <sup>b</sup> (0.38)	3.33 <sup>b</sup> (0.52)	3.86 <sup>b</sup> (1.07)	3.33 <sup>a</sup> (0.52)	1.14 (0.38)	1.00 (0.00)	2.14 <sup>b</sup> (1.07)	1.00 <sup>c</sup> (0.00)	1.00 <sup>b</sup> (0.00)	1.00 <sup>b</sup> (0.00)	1.14 (0.38)	1.00 (0.00)
Strain	**		**		ns		*		*		ns		**		**		ns	
Age	ns		**		ns		**		*		ns		*		**		ns	
Strain Age	**		ns		ns		ns		ns		ns		*		**		ns	

**Table 7:** Influence of group and age on ultrastructural eggshell quality variables in the laying hen.

(st.dev) = standard deviation of the mean; a-d = means with same superscript do not differ significantly ( $p < 0.05$ );

\*\*  $p < 0.01$ ; \*  $p < 0.05$ ; ns not statistically significant.

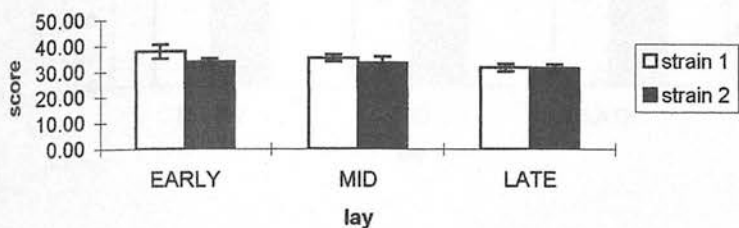
VARIABLE	CAPS		TYPE A		CHANGED MEMBRANE		TOTAL		TOTAL THICKNESS (mm)		MAMMILLARY THICKNESS (mm)		EFFECTIVE THICKNESS (mm)	
	1	2	1	2	1	2	1	2	1	2	1	2	1	2
STRAIN														
EARLY (st.dev)	4.46 <sup>a</sup> (0.78)	4.67 <sup>a</sup> (0.52)	1.00 (0.00)	1.00 (0.00)	1.92 <sup>a</sup> (0.28)	2.11 <sup>a</sup> (0.64)	37.85 <sup>a</sup> (2.67)	33.78 <sup>b</sup> (1.41)	0.309 <sup>a</sup> (0.02)	0.308 <sup>ab</sup> (0.02)	0.055 <sup>a</sup> (0.01)	0.039 <sup>b</sup> (0.01)	0.258 <sup>a</sup> (0.02)	0.273 <sup>a</sup> (0.02)
MID (st.dev)	4.77 <sup>a</sup> (0.60)	4.00 <sup>ab</sup> (0.71)	1.00 (0.00)	1.00 (0.00)	1.39 <sup>b</sup> (0.51)	1.60 <sup>ab</sup> (0.55)	35.23 <sup>b</sup> (1.42)	33.2 <sup>bc</sup> (2.59)	0.305 <sup>a</sup> (0.02)	0.282 <sup>ab</sup> (0.01)	0.047 <sup>a</sup> (0.01)	0.038 <sup>ab</sup> (0.00)	0.257 <sup>ac</sup> (0.02)	0.245 <sup>bc</sup> (0.01)
LATE (st.dev)	3.57 <sup>b</sup> (0.79)	3.50 <sup>b</sup> (1.38)	1.00 (0.00)	1.00 (0.00)	1.71 <sup>ab</sup> (0.76)	1.50 <sup>a</sup> (0.55)	31.57 <sup>c</sup> (1.40)	31.33 <sup>c</sup> (1.51)	0.310 <sup>a</sup> (0.02)	0.270 <sup>b</sup> (0.03)	0.050 <sup>a</sup> (0.01)	0.040 <sup>b</sup> (0.01)	0.260 <sup>ac</sup> (0.01)	0.234 <sup>bc</sup> (0.03)
Strain	ns		ns		ns		**		**		**		ns	
Age	**		ns		*		**		ns		ns		ns	
Strain/Age	ns		ns		ns		*		ns		ns		*	

**Table 7 (continued):** Influence of group and age on ultrastructural eggshell quality variables in the laying hen.

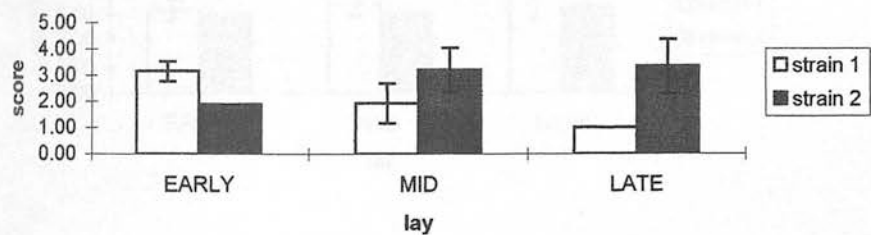
(st.dev) = standard deviation of the mean; a-d = means with same superscript do not differ significantly ( $p < 0.05$ );

\*\*  $p < 0.01$ ; \*  $p < 0.05$ ; ns not statistically significant.

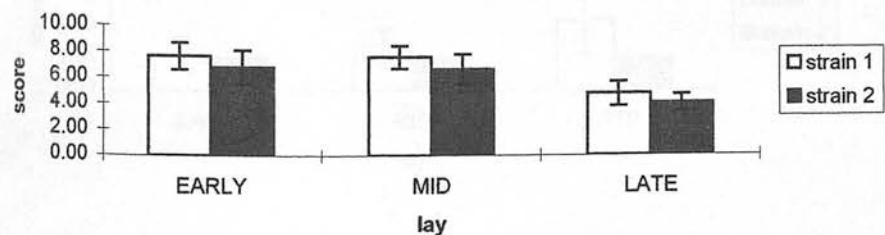




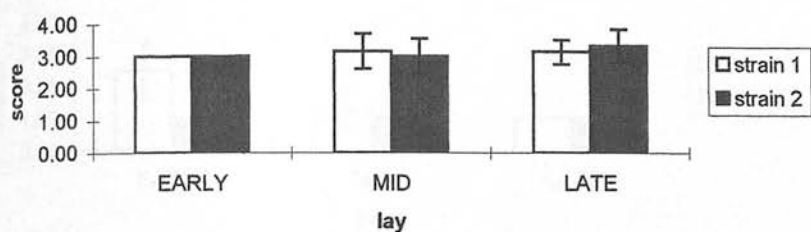
**Figure 24:** Mean total score over lay incorporating standard deviation.



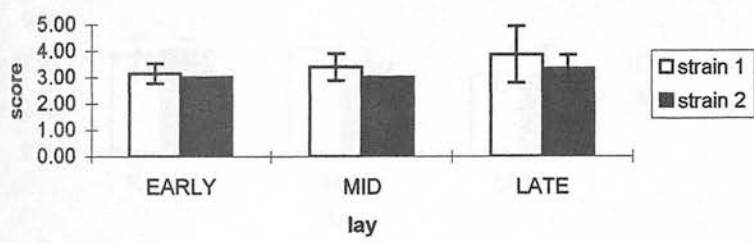
**Figure 25:** Mean confluence score over lay incorporating standard deviation (where no bar shown standard deviation = 0).



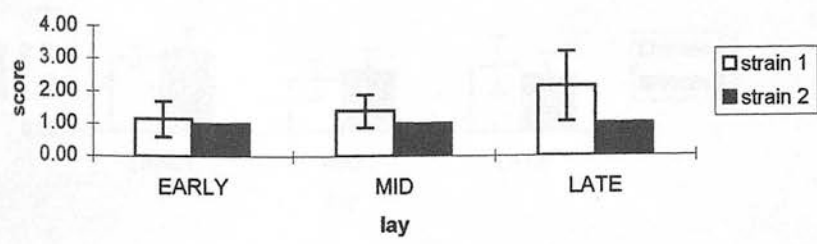
**Figure 26:** Mean fusion score over lay incorporating standard deviation.



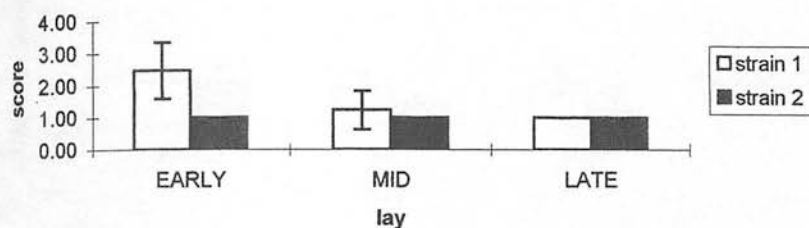
**Figure 27:** Mean alignment score over lay incorporating standard deviation(where no bar shown standard deviation = 0).



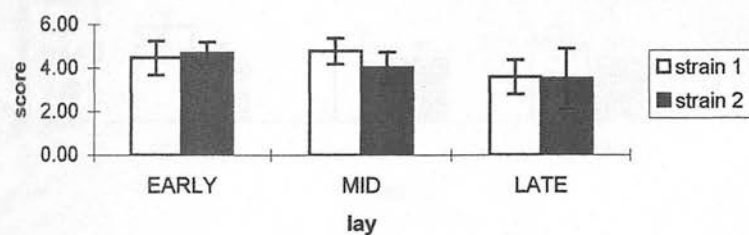
**Figure 28:** Mean type B score over lay incorporating standard deviation(where no bar shown standard deviation = 0).



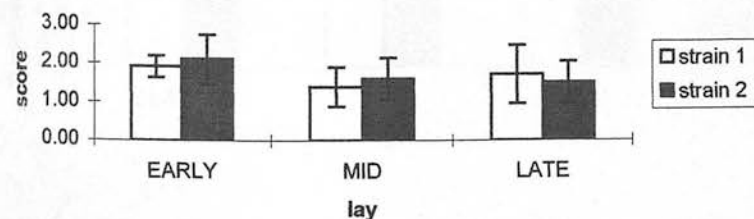
**Figure 29:** Mean erosion score over lay incorporating standard deviation(where no bar shown standard deviation = 0).



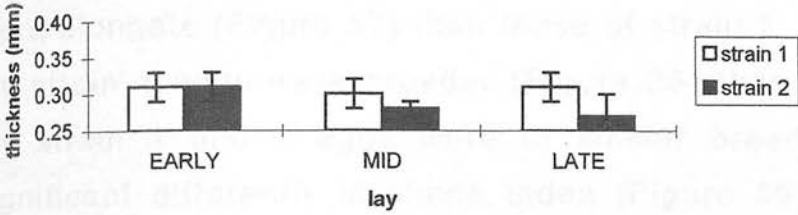
**Figure 30:** Mean cubic score over lay incorporating standard deviation (where no bar shown standard deviation = 0).



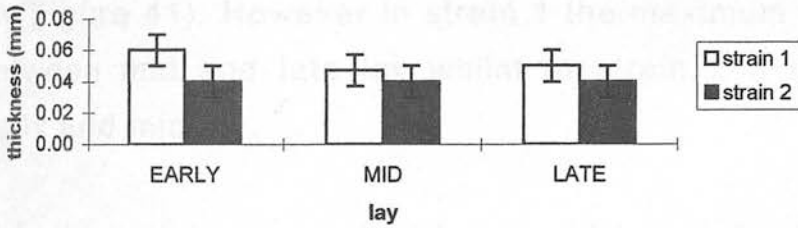
**Figure 31:** Mean caps score over lay incorporating standard deviation.



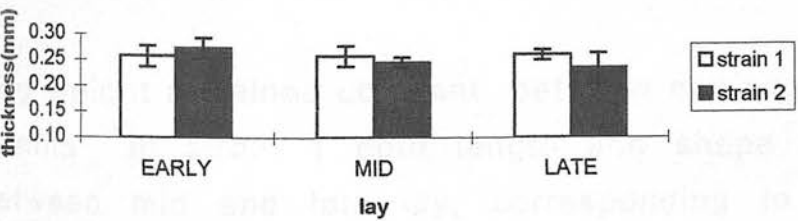
**Figure 32:** Mean changed membrane score over lay incorporating standard deviation.



**Figure 33:** Mean total thickness over lay incorporating standard deviation.



**Figure 34:** Mean mammary thickness over lay incorporating standard deviation.



**Figure 35:** Mean effective thickness over lay incorporating standard deviation.

### **iii) Traditional measures.**

The eggs of Strain 1 were consistently heavier (Figure 36) and more elongate (Figure 37) than those of strain 2. At early and mid lay strain 1 eggs were broader (Figure 38) than strain 2. At late lay strain 1 and 2 eggs were of similar breadth leading to a significant difference in shape index (Figure 39) in both at this time. There were no recorded significant differences between the two strains in terms of deformation, although evidence pointed to an increase in this measure across lay (Figure 40). At early lay the eggs of strain 2 were significantly stronger than those of strain 1. Shell strength then declined in both strains to the end of lay (Figure 41). However in strain 1 the maximum decline occurred between mid and late lay whilst in strain 2 it occurred between early and mid lay.

In both strains, egg weight increased between early and mid lay. In strain 1 this was accompanied by an increase in both length and breadth. In strain 2 length and breadth remained constant between early and mid lay, whilst stiffness (Figure 42) and breaking strength both decreased.

Egg weight remained constant between mid and late lay in both strains. In strain 1 both length and shape index increased between mid and late lay, corresponding to a decrease in breaking strength in eggs of this strain. The eggs of strain 2 demonstrated an increase in length between mid and late lay but with no subsequent change in breadth or shape index.

#### **iv) Material properties of the eggshell.**

The elastic modulus remained constant between strains and across lay. The eggs of strain 2 demonstrated significantly higher fracture toughness values (Figure 43) at both early and late lay compared to strain 1 eggs. Fracture toughness declined across lay in both strains, however in strain 1 the decline was most apparent between mid and late lay whilst in strain 2 it occurred throughout the study period.

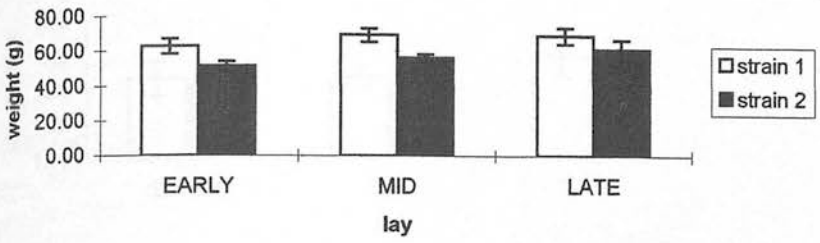


VARIABLE	EGG WEIGHT (g)		EGG LENGTH (mm)		EGG BREADTH (mm)		SHAPE INDEX		DEFORMATION ( $\mu$ m)		STRENGTH (N)		STIFFNESS (N/mm)		ELASTIC MODULUS (Nm m <sup>-2</sup> )		FRACTURE TOUGHNESS (Nm m <sup>-3/2</sup> )	
	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
STRAIN																		
EARLY (st.dev)	62.80 <sup>a</sup> (4.38)	51.24 <sup>a</sup> (3.00)	57.7 <sup>a</sup> (1.73)	53.9 <sup>c</sup> (1.54)	43.6 <sup>a</sup> (1.13)	41.3 <sup>d</sup> (1.03)	1.33 <sup>a</sup> (0.05)	1.31 <sup>a</sup> (0.04)	19.0 <sup>a</sup> (3.20)	20.5 <sup>a</sup> (4.13)	30.7 <sup>a</sup> (5.63)	38.1 <sup>b</sup> (4.05)	189 <sup>a</sup> (34.36)	204 <sup>a</sup> (19.43)	35883 (6610)	36042 (4971)	608 <sup>a</sup> (154.09)	758 <sup>b</sup> (106.38)
MID (st.dev)	69.40 <sup>b</sup> (3.90)	56.39 <sup>c</sup> (2.27)	59.9 <sup>b</sup> (2.38)	55.2 <sup>c</sup> (1.74)	45.0 <sup>b</sup> (1.00)	42.5 <sup>cd</sup> (0.34)	1.33 <sup>a</sup> (0.03)	1.30 <sup>a</sup> (0.04)	21.1 <sup>a</sup> (3.06)	21.9 <sup>ab</sup> (0.83)	31.4 <sup>a</sup> (2.73)	29.4 <sup>a</sup> (1.98)	195 <sup>ab</sup> (31.96)	165 <sup>b</sup> (16.53)	38121 (6281)	32638 (2073)	629 <sup>a</sup> (83.07)	632 <sup>ab</sup> (29.04)
LATE (st.dev)	69.20 <sup>b</sup> (4.64)	61.39 <sup>c</sup> (5.67)	62.1 <sup>c</sup> (2.63)	58.13 <sup>b</sup> (2.12)	44.7 <sup>ab</sup> (1.03)	43.8 <sup>bc</sup> (1.16)	1.39 <sup>b</sup> (0.06)	1.33 <sup>c</sup> (0.02)	22.1 <sup>ab</sup> (2.60)	25.4 <sup>b</sup> (6.77)	22.4 <sup>bc</sup> (4.45)	26.8 <sup>ac</sup> (5.10)	182 <sup>ab</sup> (36.46)	151 <sup>b</sup> (31.38)	34750 (4778)	34240 (3282)	420 <sup>b</sup> (63.90)	599 <sup>a</sup> (56.52)
Strain	**		**		**		**		ns		*		ns		ns		**	
Age	**		**		**		*		*		**		*		ns		**	
Strain Age	ns		ns		ns		ns		ns		*		*		ns		ns	

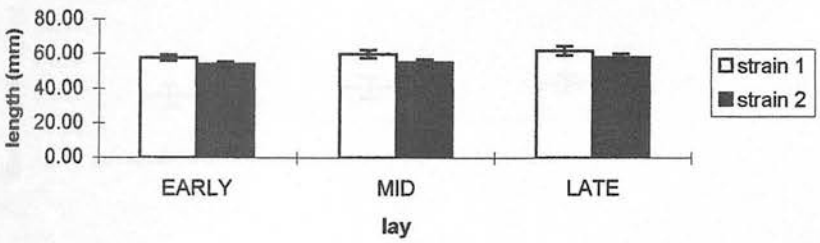
**Table 8:** Influence of strain and age on egg variables in the laying hen.

(st.dev) = standard deviation of the mean; a-d = means with same superscript do not differ significantly ( $p < 0.05$ );

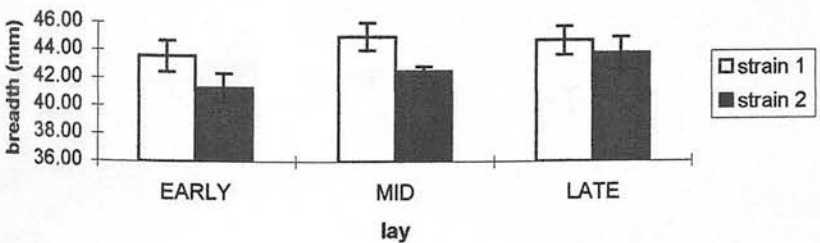
\*\*  $p < 0.01$ ; \*  $p < 0.05$ ; ns not statistically significant.



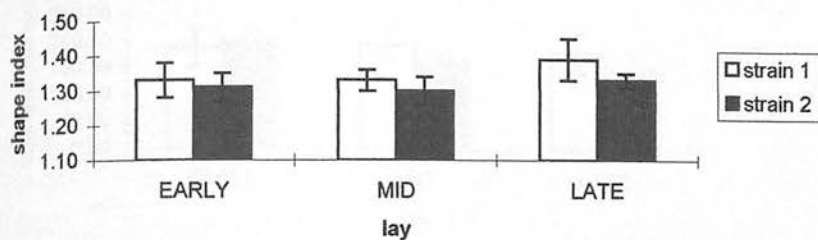
**Figure 36:** Mean egg weight over lay incorporating standard deviation.



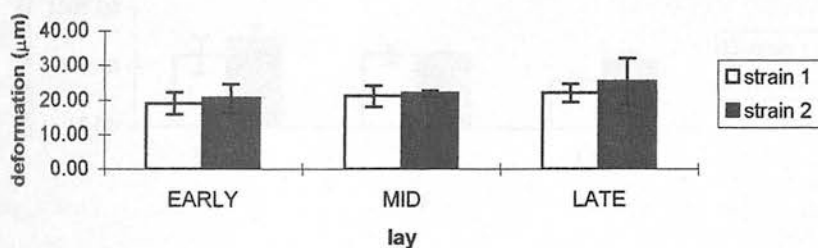
**Figure 37:** Mean egg length score over lay incorporating standard deviation.



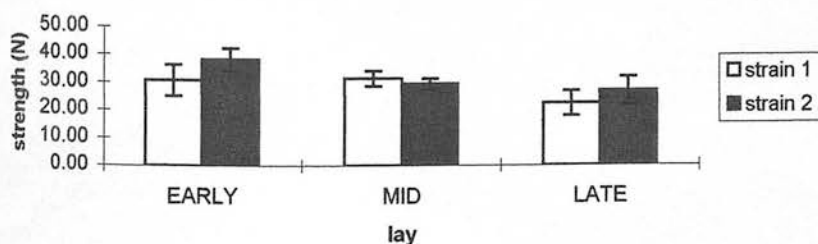
**Figure 38:** Mean egg breadth over lay incorporating standard deviation.



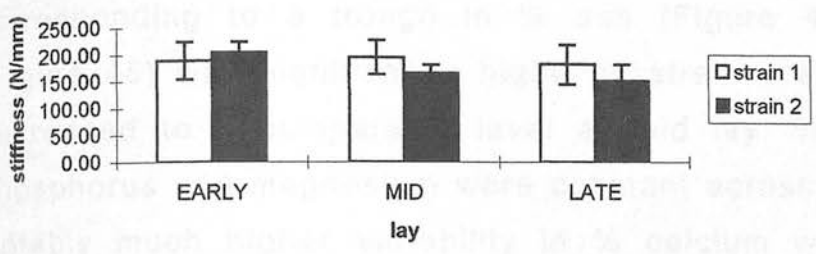
**Figure 39:** Mean shape index over lay incorporating standard deviation.



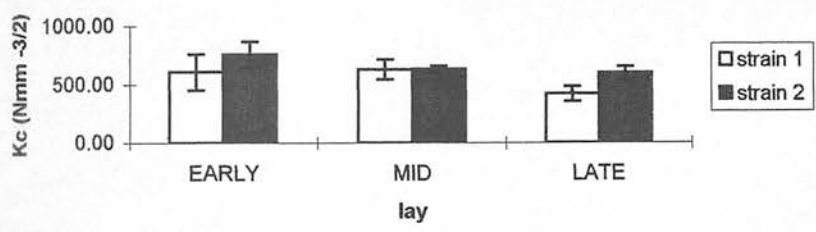
**Figure 40:** Mean deformation over lay incorporating standard deviation.



**Figure 41:** Mean breaking strength over lay incorporating standard deviation.



**Figure 42:** Mean stiffness over lay incorporating standard deviation.



**Figure 43:** Mean fracture toughness over lay incorporating standard deviation.

**v) Eggshell mineral analysis.** Strain 1 demonstrated peaks of phosphorus (Figure 46) and magnesium (Figure 47) at mid lay, corresponding to a trough in % ash (Figure 44). % calcium (Figure 45) was significantly higher in strain 1 at early lay, but decreased to a comparable level at mid lay. % ash, calcium, phosphorus and magnesium were constant across lay in strain 2. Notably much higher variability in % calcium was recorded in strain 2, particularly at mid and late lay, inferring a lack of uniformity in the eggs produced.

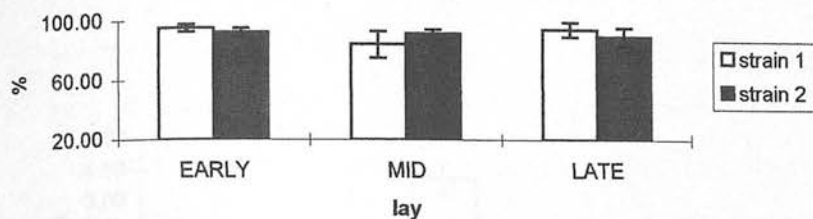
VARIABLE	%ASH (dry fat free)		% CALCIUM		% PHOSPHORUS		% MAGNESIUM	
	1	2	1	2	1	2	1	2
STRAIN								
EARLY	95.2 <sup>a</sup>	92.19 <sup>a</sup>	44.97 <sup>a</sup>	37.92 <sup>b</sup>	0.06 <sup>a</sup>	0.09 <sup>a</sup>	0.59 <sup>a</sup>	0.57 <sup>a</sup>
(st.dev)	(2.52)	(3.18)	(9.27)	(8.37)	(0.03)	(0.03)	(0.18)	(0.21)
MID	84.17 <sup>b</sup>	91.66 <sup>a</sup>	34.32 <sup>b</sup>	37.38 <sup>b</sup>	0.31 <sup>b</sup>	0.10 <sup>a</sup>	3.73 <sup>b</sup>	0.82 <sup>a</sup>
(st.dev)	(8.87)	(2.99)	(2.28)	(8.63)	(0.13)	(0.02)	(0.50)	(0.30)
LATE	94.66 <sup>a</sup>	89.72 <sup>a</sup>	32.71 <sup>b</sup>	39.38 <sup>b</sup>	0.07 <sup>a</sup>	0.07 <sup>a</sup>	0.50 <sup>a</sup>	0.69 <sup>a</sup>
(st.dev)	(4.96)	(6.10)	(4.83)	(8.37)	(0.02)	(0.03)	(0.09)	(0.15)
Strain	ns	ns	ns	ns	ns	ns	**	**
Age	ns	ns	ns	ns	**	**	**	**
Strain/Age	*	*	*	*	*	*	**	**

**Table 9:** Influence of strain and age on eggshell mineral in the laying hen.

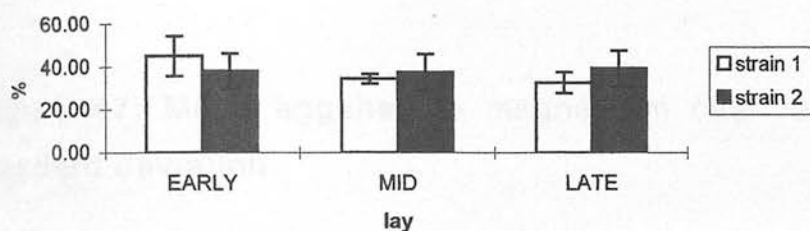
(st.dev) = standard deviation of the mean; a-c = means with same superscript do not differ significantly ( $p < 0.05$ );

\*\*  $p < 0.01$ ; \*  $p < 0.05$ ; ns not statistically significant.

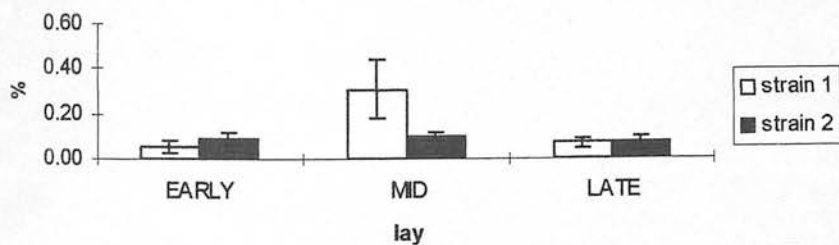




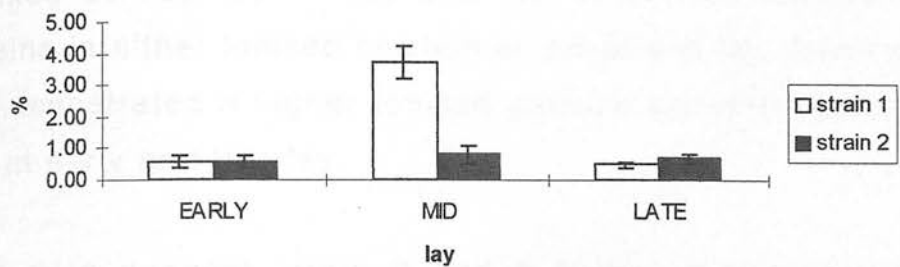
**Figure 44:** Mean eggshell % ash over lay incorporating standard deviation.



**Figure 45:** Mean eggshell % calcium over lay incorporating standard deviation.



**Figure 46:** Mean eggshell % phosphorus over lay incorporating standard deviation.



**Figure 47:** Mean eggshell % magnesium over lay incorporating standard deviation.

**vi) Blood samples.** In both strains ionised calcium (Figure 48) displayed a trough at mid lay in contrast to pH (Figure 49) which peaked at mid lay. There was no difference between the two strains in either ionised calcium or pH at mid lay. However, strain 2 demonstrated a higher ionised calcium concentration and lower pH at early and late lay.

The data suggest strain 2 had a higher concentration of total calcium (Figure 50) throughout the study period. Strain 2 displayed a significantly higher concentration of phosphorus (Figure 51) at mid lay. Magnesium levels remained comparable throughout. Total calcium concentration remained constant across lay in both strains, whilst phosphorus concentration decreased.

Strain 1 demonstrated higher alkaline phosphatase (Figure 52) concentrations at mid and late lay, whilst strain 2 displayed higher osteocalcin (Figure 53) concentrations across lay. In strain 1 alkaline phosphatase levels remained constant across lay while osteocalcin levels increased between mid and late lay. In strain 2 alkaline phosphatase decreased across lay and osteocalcin increased from early to mid lay.

VARIABLE	IONISED CALCIUM		pH		TOTAL CALCIUM		TOTAL PHOSPHORUS		TOTAL MAGNESIUM		ALKALINE PHOSPHATASE		OSTEOCALCIN	
	1	2	1	2	1	2	1	2	1	2	1	2	1	2
STRAIN														
EARLY (st.dev)	1.56 <sup>a</sup> (0.12)	1.69 <sup>c</sup> (0.08)	7.38 <sup>a</sup> (0.06)	7.30 <sup>c</sup> (0.05)	5.72 <sup>a</sup> (1.92)	7.14 <sup>b</sup> (1.08)	13.73 <sup>ac</sup> (8.17)	15.09 <sup>c</sup> (3.80)	3.72 (3.74)	1.94 (0.44)	13.48 <sup>ab</sup> (4.96)	9.95 <sup>b</sup> (5.49)	3.80 <sup>a</sup> (3.82)	23.90 <sup>c</sup> (15.69)
MID (st.dev)	1.40 <sup>b</sup> (0.09)	1.45 <sup>b</sup> (0.12)	7.50 <sup>b</sup> (0.07)	7.51 <sup>b</sup> (0.05)	5.54 <sup>a</sup> (1.70)	7.15 <sup>b</sup> (1.29)	8.4 <sup>b</sup> (4.78)	18.74 <sup>c</sup> (8.29)	1.62 (0.45)	1.52 (0.51)	13.59 <sup>a</sup> (5.99)	6.11 <sup>bc</sup> (2.48)	7.66 <sup>a</sup> (6.96)	43.08 <sup>d</sup> (11.12)
LATE (st.dev)	1.44 <sup>b</sup> (0.13)	1.62 <sup>c</sup> (0.12)	7.41 <sup>a</sup> (0.04)	7.34 <sup>c</sup> (0.05)	6.45 <sup>a</sup> (2.62)	7.78 <sup>b</sup> (1.85)	7.7 <sup>b</sup> (3.66)	8.99 <sup>b</sup> (6.14)	2.98 (2.62)	4.13 (3.58)	13.44 <sup>a</sup> (4.23)	3.88 <sup>c</sup> (0.98)	20.4 <sup>b</sup> (11.87)	36.14 <sup>cd</sup> (8.79)
Strain	**		**		**		*		ns		**		*	
Age	**		ns		**		*		ns		*		*	
Strain Age	ns		ns		ns		ns		ns		**		ns	

**Table 10:** Influence of strain and age on blood parameters in the laying hen.

(st.dev) = standard deviation of the mean; a-d = means with same superscript do not differ significantly ( $p < 0.05$ );

\*\*  $p < 0.01$ ; \*  $p < 0.05$ ; ns not statistically significant.

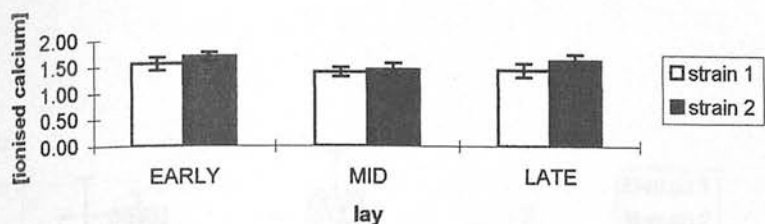


Figure 48: Mean blood ionised calcium over lay incorporating standard deviation.

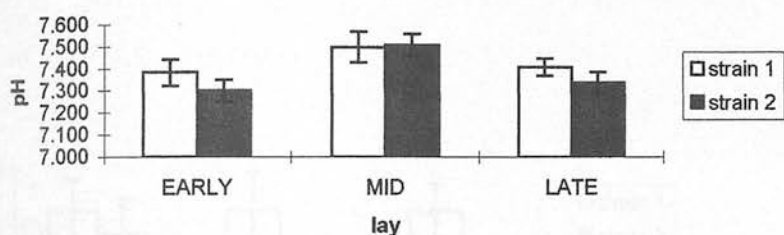


Figure 49: Mean blood pH over lay incorporating standard deviation.

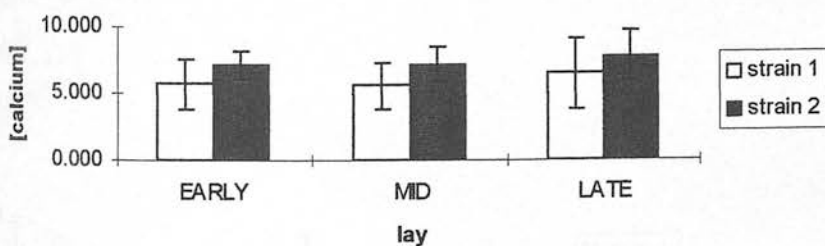


Figure 50: Mean plasma total calcium over lay incorporating standard deviation.

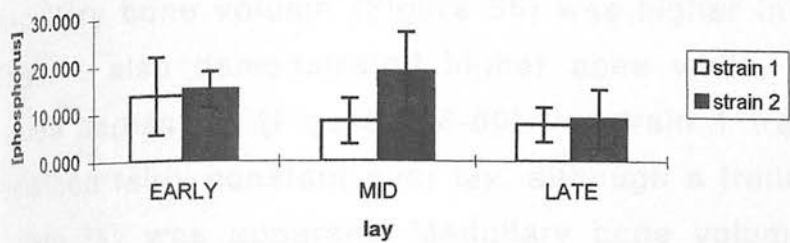


Figure 51: Mean plasma phosphorus over lay incorporating standard deviation.

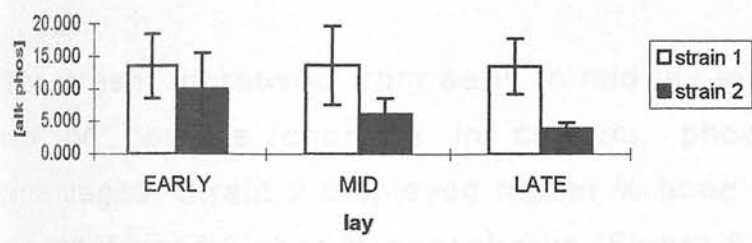


Figure 52: Mean plasma alkaline phosphatase over lay incorporating standard deviation.

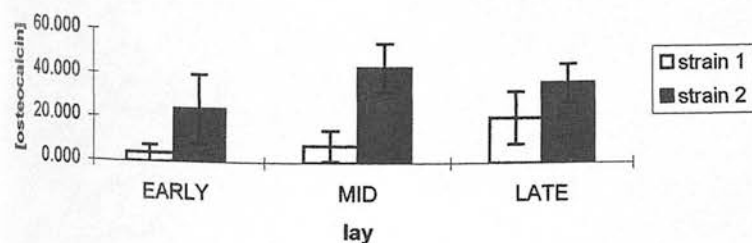


Figure 53: Mean plasma osteocalcin over lay incorporating standard deviation.



vii) **Bone histomorphometry.** Trabecular bone volume (Figure 54) and bone density (Figure 56) were consistently higher in strain 2 while medullary bone volume (Figure 55) was higher in strain 1 (Figure 57). Strain 1 also demonstrated higher bone width, marrow width and K values across lay (Figures 58-60). In strain 1 trabecular bone volume remained fairly constant over lay, although a trend towards a decrease at late lay was apparent. Medullary bone volume increased between early and mid lay in strain 1 and between mid and late lay in strain 2. In strain 2 there was a significant decrease in trabecular bone volume across lay, with high variability at early lay. However medullary bone volume remained constant. Bone density increased across lay in strain 2.

% bone ash increased from early to mid lay in strain 1, however there were no obvious changes in calcium, phosphorus or magnesium percentages. Strain 2 displayed higher % bone ash (Figure 61) at early and mid lay and higher % phosphorus (Figure 62) at early lay compared to strain 1. Variability in mineral composition increased at late lay in both strains.

VARIABLE	TRABECULAR BONE VOLUME		MEDULLARY BONE VOLUME		BONE DENSITY		MARROW WIDTH (mm)		BONE WIDTH (mm)		K	
	1	2	1	2	1	2	1	2	1	2	1	2
STRAIN												
EARLY (st.dev)	10.89 <sup>a</sup> (3.34)	26.14 <sup>b</sup> (12.21)	11.31 <sup>a</sup> (1.71)	5.98 <sup>b</sup> (4.74)	1.19 <sup>a</sup> (0.16)	1.43 <sup>b</sup> (0.15)	7.62 <sup>a</sup> (0.25)	6.52 <sup>b</sup> (0.34)	8.30 <sup>a</sup> (0.25)	7.20 <sup>b</sup> (0.31)	0.92 <sup>a</sup> (0.01)	0.90 <sup>b</sup> (0.02)
MID (st.dev)	11.42 <sup>a</sup> (4.85)	20.31 <sup>bc</sup> (4.48)	15.06 <sup>b</sup> (4.95)	3.54 <sup>a</sup> (2.41)	1.14 <sup>a</sup> (0.14)	1.60 <sup>bc</sup> (0.30)	7.75 <sup>a</sup> (0.33)	6.23 <sup>b</sup> (0.25)	8.46 <sup>a</sup> (0.29)	6.95 <sup>b</sup> (0.28)	0.92 <sup>a</sup> (0.02)	0.90 <sup>b</sup> (0.01)
LATE (st.dev)	7.66 <sup>a</sup> (4.07)	16.90 <sup>c</sup> (3.28)	17.45 <sup>b</sup> (8.86)	9.25 <sup>c</sup> (4.05)	1.14 <sup>a</sup> (0.18)	1.72 <sup>c</sup> (0.14)	7.81 <sup>a</sup> (0.40)	6.58 <sup>b</sup> (0.40)	8.54 <sup>a</sup> (0.30)	7.28 <sup>b</sup> (0.43)	0.91 <sup>ab</sup> (0.01)	0.90 <sup>b</sup> (0.01)
Strain	**		**		**		**		*		**	
Age	*		**		ns		ns		ns		ns	
Strain Age	ns		ns		*		ns		ns		ns	

**Table 11:** Influence of strain and age on bone parameters in the laying hen.

(st.dev) = standard deviation of the mean; a-d = means with same superscript do not differ significantly ( $p < 0.05$ );

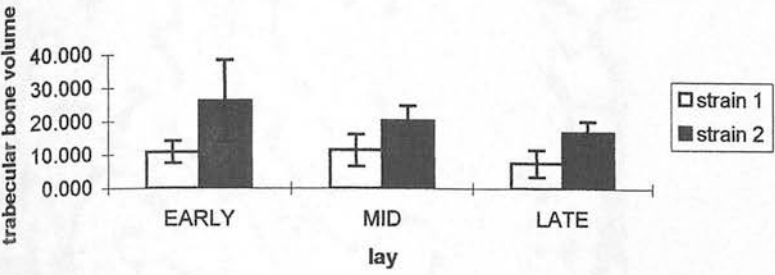
\*\*  $p < 0.01$ ; \*  $p < 0.05$ ; ns not statistically significant.

VARIABLE	%ASH (dry fat free)		% CALCIUM		% PHOSPHORUS		% MAGNESIUM	
	1	2	1	2	1	2	1	2
STRAIN								
EARLY (st.dev)	56.88 <sup>a</sup> (4.46)	60.43 <sup>c</sup> (1.80)	21.84 (5.68)	27.65 (13.08)	5.64 <sup>a</sup> (1.21)	8.57 <sup>b</sup> (2.93)	0.52 (0.11)	0.73 (0.37)
MID (st.dev)	59.06 <sup>b</sup> (2.22)	65.99 <sup>c</sup> (2.64)	26.24 (10.99)	27.03 (4.96)	8.23 <sup>a</sup> (1.45)	9.00 <sup>ab</sup> (2.40)	0.55 (0.20)	0.60 (0.15)
LATE (st.dev)	60.90 <sup>bc</sup> (5.56)	62.75 <sup>c</sup> (6.08)	22.62 (8.71)	16.45 (7.28)	6.95 <sup>a</sup> (2.00)	7.48 <sup>ab</sup> (3.85)	0.53 (0.19)	0.48 (0.13)
Strain	**		ns		*		ns	
Age	*		ns		ns		ns	
Strain/Age	ns		ns		ns		ns	

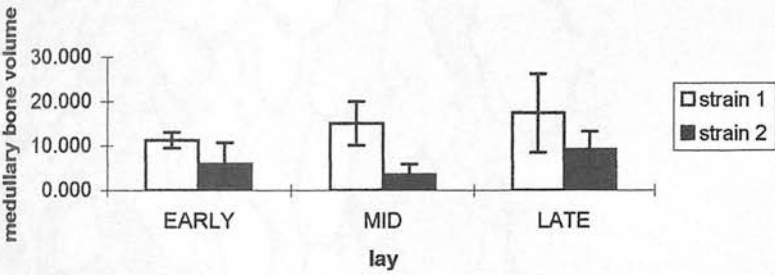
**Table 11 (continued):** Influence of group and age on bone parameters in the laying hen.

(st.dev) = standard deviation of the mean; a-c = means with same superscript do not differ significantly ( $p < 0.05$ );

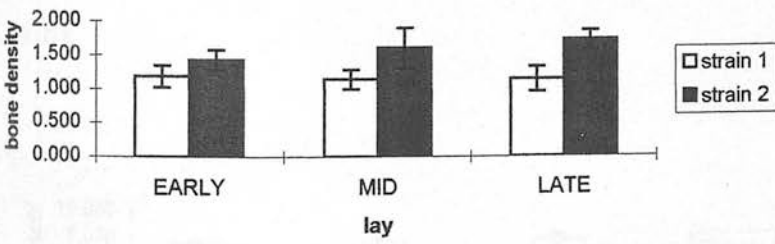
\*\*  $p < 0.01$ ; \*  $p < 0.05$ ; ns not statistically significant.



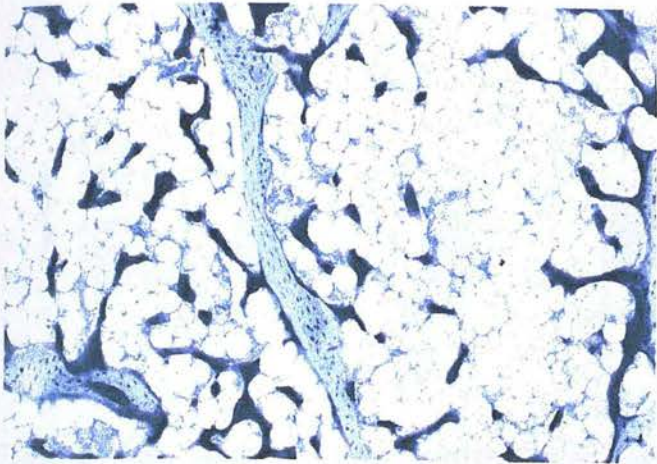
**Figure 54:** Mean trabecular bone volume over lay incorporating standard deviation.



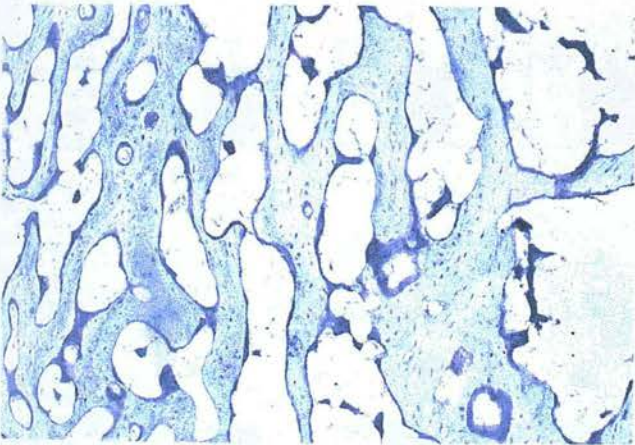
**Figure 55:** Mean medullary bone volume over lay incorporating standard deviation.



**Figure 56:** Mean bone density over lay incorporating standard deviation.



Commercial strain (Strain 1)



J.Line Strain (Strain 2)

Figure 57: Comparison of bone histomorphometry between strains.

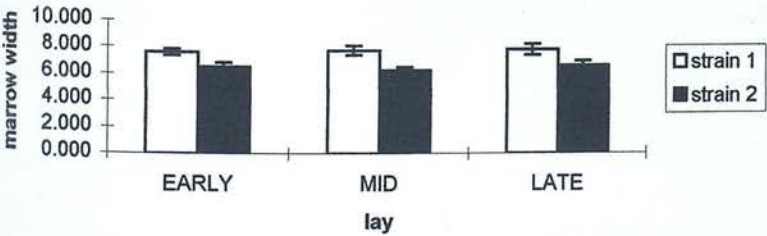


Figure 58: Mean marrow width over lay incorporating standard deviation.

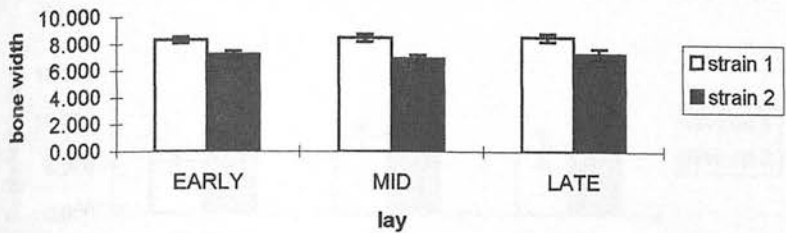


Figure 59: Mean bone width over lay incorporating standard deviation.

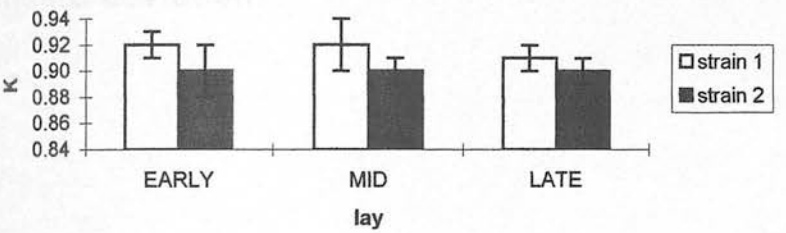


Figure 60: Mean K over lay incorporating standard deviation.

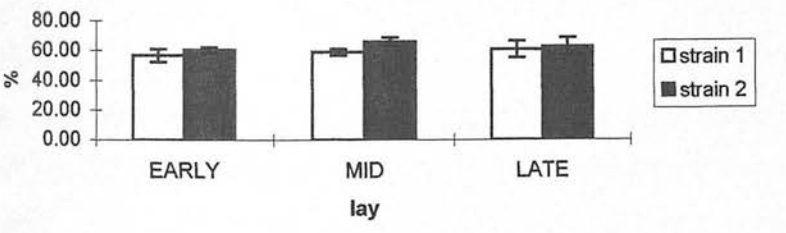
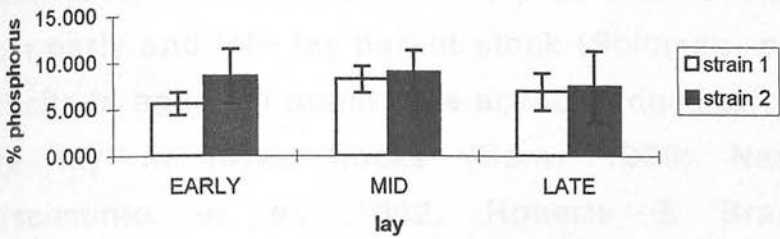


Figure 61: Mean bone % ash over lay incorporating standard deviation.



2.4 DISCUSSION



**Figure 62:** Mean bone % phosphorus over lay incorporating standard deviation.

### 3.4 DISCUSSION

The processes of bone remodelling and eggshell calcification are inextricably linked. Chick quality problems have been documented from early and late lay parent stock (Solomon *pers comm*) whilst deficits in eggshell quality are acknowledged to occur at early and late lay in layer flocks (Bain, 1990; Nascimento, 1990; Nascimento *et al*, 1992; Roberts & Brackpool, 1993-4) accompanied by a high incidence of bone breakages at late lay (Gregory & Wilkins, 1989 & 1992; Hughes *et al*, 1995). Previous authors have correlated such bone disorders with the high demand for calcium required by eggshell calcification (Riddell, 1989) and the length of continuous reproduction (Whitehead & Wilson, 1992; Rennie *et al*, 1997). However little investigation has been carried out on the relationship between bone histomorphometry and eggshell quality.

This chapter compared eggshell quality and bone histomorphometry in a highly selected commercial line and the J.Line, a relatively unselected Brown leghorn strain. This comparison took place within the previously identified 'problem areas' in commercial poultry keeping; for example at hatch, early and late lay. A mid-lay sample was also included for the purposes of continuity. Rennie *et al* (1997) described the J.Line as not experiencing structural bone loss during lay due to its relatively low egg output. Thus, the J.Line provided a good model with which to compare today's commercial hybrid.

The embryonic chick, as with the human embryo, relies upon its mother to supply the requirements for development and protect it from the harsh external environment, the difference being that in the avian this is achieved outwith the body. The majority of the mineral requirements for embryonic development are produced by

the mother in 23 hours. Bray & Iton (1962) demonstrated the effect of parent weight on embryo weight whilst Davis Buckner *et al* (1926) produced significant differences in embryonic development through maternal calcium carbonate supplementation and housing condition, both of which have been demonstrated to affect eggshell quality in the laying hen (Hughes *et al*, 1995). Further Munro (1940) stated that 'eggs from periods of inefficient physiological function will be of poor hatching quality'. Thus the requirement for a structurally sound eggshell becomes apparent. As described in the introduction, the mineralisation of the developing skeletal system occurs during the last 10 days of incubation through the calcium dependent processes of intramembranous and endochondrial ossification (Gay, 1988). 80% of the total calcium content of the chick is eggshell sourced (Simkiss, 1961). The 'placenta' of the embryonic chick is the eggshell membrane which depends on good contact with the mammillary layer of the eggshell. Roberts *et al* (1992) demonstrated the presence of a range of ultrastructural variations similar to that in a whole egg, within the hatched egg. Ultrastructural variations observed in successfully hatched eggshells were quantified in this chapter utilising a modified form of the scoring system previously applied to table eggs (Reid, 1984; Watt, 1985, 1989; Bain, 1990; Solomon, 1991; Fraser, 1996).

Upon hatching, the female chicks of both the selected commercial (Strain 1) and unselected (Strain 2) lines did not differ in terms of the mineral composition of their femora. Similarly the results of the analysis of ultrastructural detail demonstrated no differences in the composition of the mammillary layer in terms of the variants which would be likely to affect mineral uptake from the eggshell (for example, cubics, type A bodies, type B Bodies, changed membrane, confluence). In contrast, the total ultrastructural

eggshell quality score (a summation of all the individual values) did vary at the level of the mammillary layer, this difference being accounted for by the high incidence of early fusion and cuffing in the unselected Strain 2. Both of these ultrastructural variants lead to an effectively thicker eggshell. Although minor relative to the thickness of the entire eggshell, Bain (1990) has demonstrated a relationship between early fusion and crack propagation in table eggs. Within the hatching unselected (strain 2) egg the high incidence of early fusion and cuffing may be partly responsible for the low hatchability observed in the J.Line (70-80%, Roslin Nutrition *pers comm*). After all the mammillary layer is the primary obstacle which must be overcome in order to assist pipping.

In absolute terms the eggshells of the selected commercial strain demonstrated a greater remaining, mammillary and effective thickness, reflecting current commercial genetic selection criteria. O'Neil & Spinks (1953) have previously demonstrated a significant relationship between eggshell thickness and calcium utilisation. Within the unselected J.Line strain a significant positive relationship was apparent between mammillary thickness and the % bone ash from the femur. Also within this strain, hatched eggshells of a greater effective thickness tended to have a higher magnesium content. Magnesium is present within both the outer layers of the eggshell (Brooks & Hale, 1955; Itoh & Hatano, 1964) and at the level of the mammillary bodies (Solomon, 1986). However, its role in eggshell quality remains to be established. Magnesium supplementation is not routinely afforded as it is generally assumed that this element is adequately provided for in the diet. Waddell *et al* (1989) demonstrated both a reduction in the number of eggs laid and the quality of eggs, from hens fed on a diet containing less than 0.21% magnesium. Within the J.Line strain, eggshells

demonstrating high % magnesium lead to chicks with low % magnesium. Given the diverse location of this element within the eggshell, and in the absence of any evidence to suggest that its positioning is shared equally between the upper and lower regions of the shell thickness, it might be hypothesised that in this instance magnesium was primarily located in the upper palisade layer and so unavailable to the developing embryo.

Previous work has demonstrated little difference in total eggshell thickness after hatch (Bennet, 1995). It is of interest to note that in the experiments measured by this author total thickness was conducted by a fairly gross method with the aid of a micrometer, such measurements are inadequate to detect the subtle changes which occur during hatching. Both the effective and mamillary thickness of the J.Line hatched eggshells in this experiment were reduced when compared to those of a similar age (see 3.3.2ii). However little difference was apparent in total thickness. No comparison can be made between hatched and table eggshells in the commercial strain as breeder and layer hens have different performance characteristics.

The remaining chicks hatched at the beginning of these experiments were allowed to mature into layers in order to compare the relationship between eggshell quality and bone morphology throughout lay. Therefore, in the interim, they had undergone further bone growth. Approximately 14-16 days prior to the onset of lay medullary bone formation commences (Hurwitz, 1964) to produce a labile calcium source for eggshell calcification (see Chapter 4). Experimentation then commenced at early lay, as defined by eggshell quality.



Eggshell quality at an individual bird level is highly variable (Belyavin *et al*, 1985) and so taking one egg as representative of that individual's performance is erroneous. For this reason eggs were collected over a period of 14 days from each individual bird, in order to provide a measure which could then be related to the bone parameters subsequently measured on that individual. The assessment of traditional measures, material properties and mineral composition were carried out on a group basis using a subset of 1 day's production. The very nature of blood sampling is stressful and care must be taken to minimise trauma within the experimental set up. It was therefore decided to blood sample, as a single event, after all egg collections were finished and immediately prior to culling the birds.

The selected commercial layers (strain 1) not surprisingly displayed a significantly higher rate of production than the unselected hen (strain 2). In both strains production decreased between the middle and end of lay. Thus peak production had been achieved by the early lay sampling period in both strains. The fact that production subsequently decreases and variability increases in the lower egg number J.Line suggests that there must exist a physiological maximum in the laying hen, after which continued reproduction must decrease. It would appear that selection in the commercial hybrid has attempted to overcome this physiological barrier with the result that "on farm" seconds dramatically increase at late lay. Egg weight (g/hen/day) was consistently higher in the selected commercial strain. Egg weight (g/hen/day) increased in both strains between early and mid lay in accordance with the work of Williams & Sharp (1978) who suggested that egg size increases with age due to an increase in the yolk accumulation per follicle.



Ultrastructural eggshell quality improved across lay in both strains, which is in contrast to the results of previous authors (Boorman *et al*, 1985; Watt, 1989; Bain, 1990; Roberts & Brackpool, 1993-4). However the eggshells analysed by these authors were from commercial flocks whereas the birds used in this experiment were maintained in individual cages. Thus, the relatively good quality of the eggs studied herein may reflect a lower degree of stress due to the conditions imposed in this experimental set-up. The selected commercial hybrid demonstrated significantly poorer ultrastructural eggshell quality at early lay accompanied by high inter-bird variation, when compared to the unselected line (strain 2). A high incidence of confluence and cubics was observed within the shells of the former. The ultrastructural variation termed confluence is particularly characteristic of the eggs of young birds (Solomon *pers comm*), and can lead to areas of weakness within the egg where confluent and normal areas meet. Rhombohedral calcite crystals occur in the intermammillary spaces where the latter enable calcium carbonate to precipitate in its preferential form. Thus even with increased selection for eggshell quality in today's layer flocks, there still exists a propensity for poor eggshell quality at early lay. Interestingly despite the high increase in "on farm" seconds in strain 1, there was no difference in ultrastructure between the two strains at late lay. This suggests that the bird may preferentially maintain ultrastructure at the expense of external quality as the development of the chick within the eggshell depends upon the former feature. The incidence of erosion did increase in the selected commercial line from mid to late lay, which may account for some of the increase in "on farm" seconds. This ultrastructural variation leads to areas of weakness within the eggshell contributing to crack propagation. In the J.Line there was a high rate of variation at mid lay, perhaps reflecting the physiological maximum egg number for this strain.

The selected commercial strain (strain 1) demonstrated significantly higher total thickness and mamillary thickness values at late lay and higher mamillary thickness values at early lay, cf. unselected strain (strain 2). All measures of thickness remained constant with increasing bird age in strain 1, however effective thickness decreased between early and mid lay in strain 2.

At early lay it was evident that the significantly poorer ultrastructure of the commercial hybrid egg was mirrored by a deficit in both breaking strength and fracture toughness. Thus, as suggested the high incidence of confluence at early lay in this strain may have led to areas of weakness within the eggshell. At late lay, the high incidence of "on farm" seconds in the commercial hybrid strain was again evidenced by a significant decrease in breaking strength and fracture toughness. Although such a similar decrease in fracture toughness was also apparent in the unselected strain it was of a magnitude lower than of the commercial hybrid.

The decrease in production at late lay in both strains was accompanied by increases in egg weight, size, deformation and decreased strength, stiffness and fracture toughness. The results presented herein are in agreement with those of Brooks (1971), Watt (1989) and Bain (1992) all of whom demonstrated an inverse relationship between eggshell strength and age. This may provide the reason why production slows at this time. If production was maintained the resulting physically poor eggshells would provide substandard embryonic chambers and thus reduce the amount of young going to the next generation. The previously presented data has shown that ultrastructural eggshell quality did not decrease at late lay although on farm seconds did increase in

Strain1. The poor ultrastructural eggshell quality at late lay may reflect a reduction in available calcium or a change in the eggshell matrix. Knott *et al* (1995) demonstrated significant changes in the collagen matrix of osteoporotic bone at late lay, adding to the decrease in bone strength at this time. It is therefore possible that similar changes also occur within the matrix of the egg leading to poor egg strength. Indeed Fraser *et al* (1998) have demonstrated significant changes in matrix morphology concomitant with the decline in shell quality at the end of lay.

The decrease in effective thickness between early and mid lay in the unselected J.Line strain was accompanied by a decrease in both breaking strength and stiffness.

The percentage ash composition of the eggs of the unselected strain (Strain 2) remained constant throughout the experiment, although both egg weight and eggshell thickness measures increased. From these data it can be inferred that the total amount of mineral needed per shell has subsequently increased putting more demand on the skeletal system. The selected commercial bird (strain 1) demonstrated significant changes in eggshell mineral composition over lay. Mean % ash decreased at mid lay corresponding to a decrease in % calcium and an increase in % phosphorus and magnesium. As the composition of the feed remained constant across lay it is feasible that the decrease in % calcium resulted from some deficit in bone calcium stores. The increase in % phosphorus incorporation at this time also corresponds to this hypothesis with phosphorus correcting for the deficit in calcium within the eggshell. The reason for an increase in % magnesium could be two fold. It could be replacing calcium within the eggshell or alternatively, as phosphorus has been found to decrease the strength of the eggshell (Sauveur &

Mongin, 1983; Gunaratne & Boorman, 1996), magnesium may be present in larger amounts to offset to some extent any deficits in eggshell strength at this time (Anon, 1967). Bastien *et al* (1979) found an increase in both eggshell weight and percentage shell in birds supplemented with magnesium sulphate.

% eggshell ash increased to late lay in strain 1, and both % phosphorus and magnesium decreased to early lay levels, yet % calcium remained constant. Whilst this result in itself suggests decreased levels of calcium within the bone in strain 1, it also raises the question as to what has increased to bring the % ash back to its early lay levels? Further, this continued low level of eggshell calcium may partly explain the reduction in egg strength at late lay.

The apparent high levels of % calcium in the commercial hybrid eggshell at early lay, in comparison with the J.Line, were probably due to the different mechanism of calcium procurement. Previous authors have demonstrated that the J.Line has little medullary bone (Rennie *et al*, 1997) thus all calcium is food sourced whilst the commercial hybrid is able to achieve calcification from medullary bone. The level of % calcium within the eggshell decreases to level with the J.Line at mid/late lay as bone stress increases.

During the process of eggshell calcification, the related processes of bone resorption and osteoid formation are elevated while bone mineralisation is suppressed (Van de Velde *et al*, 1985). As described in Chapter 2, this leads to high levels of circulating total calcium for incorporation into the calcifying eggshell. Chapter 2 confirmed osteocalcin as a marker of osteoid formation during eggshell calcification. The previous chapter also demonstrated the increase in inorganic phosphate accompanying



the decrease in total calcium during eggshell calcification. The current chapter focused on one time point only during calcification (i.e. 10 hours post ovulation), when both blood and bone were analysed.

The commercial selected strain (strain 1) demonstrated an increase in the mean osteocalcin concentration at late lay consistent with an increase in osteoid formation at this time. This result is compatible with a bird which is actively remodelling medullary bone over a large surface area. Riddell (1989) described the medullary bone of osteoporotic hens as being almost pure osteoid. Mean osteocalcin concentration was significantly higher in the unselected J.Line strain across lay pointing to a higher rate of osteoid formation in this strain. As there is no evidence of osteoporosis in this strain (Rennie *et al*, 1997), it can be hypothesised that the higher concentrations of osteocalcin recorded were as a result of the inter-clutch breaks, allowing more extensive bone repair. In the J.Line strain an increase in osteocalcin was recorded at mid lay, demonstrating an increase in osteoid formation, paralleling the time of egg weight increase in this strain and may reflect an increased rate of bone remodelling to support the extra mineral demands placed on the system. The magnitude of this difference in osteocalcin concentration in this experiment compared to that in chapter 2 was due to the use of different batches of labelled antibody and must be considered before undertaking comparative experiments (Williams *pers comm*).

Alkaline phosphatase is a measure of the rate of osteoid mineralisation occurring during eggshell calcification. In the selected commercial laying hybrid strain used in this experiment alkaline phosphatase remained constant across the laying period, indicating no change in the rate of osteoid mineralisation.

Nevertheless, when the results of the osteocalcin assay were put into this scenario it would appear that an increase in the rate of osteoid formation is occurring, i.e. there is no synchronisation in the rate of these two events. This would have led to a decrease in the proportion of mineralised osteoid.

In the unselected strain (strain 2) the mean concentration of alkaline phosphatase decreased across the laying period to a level lower than that recorded in the selected commercial line at mid and late lay. Thus in this strain the rate of osteoid formation is high whilst the rate of osteoid mineralisation is low. This may reflect the increased complexity of the osteoid in this strain, which previous authors have demonstrated to have low medullary bone reserves (Rennie *et al*, 1997). A decrease in mineralisation may be occurring since the J.Line is having to remodel structural bone because of a lack of medullary bone compared to the commercial hybrid, however the length of the inter-clutch break appears to prevent any deleterious effects as a result of this process.

Although remaining constant across lay in both strains, the mean concentration of total plasma calcium was significantly higher in the unselected strain (strain 2) throughout the experiment. This might indicate that the selected strain (strain 1) may have been under some stress with regards to calcium balance. As there were differences in both the size of the eggs produced and the amount of calcium present within the eggshell it is hypothesised that calcium is being resorbed from the bone at its maximal rate in the latter strain. Thus at mid lay, the increased egg size combined with high production may point to the fact that there was not sufficient calcium to cover demand. These results are in agreement with those of Gunaratne & Boorman (1996) who found no change in the plasma concentration of total calcium across



lay. Although there was no significant difference in plasma calcium between the two genetically distinct strains, this does not imply that bone histomorphometry is normal. Bell & Siller (1962) found total calcium concentration in the plasma was not significantly different in birds demonstrating severe osteoporosis.

The concentration of ionised calcium reflects that which has arrived in the bloodstream from bone derived sources. In the selected strain (strain 1) blood ionised calcium decreases from early to mid lay accompanied by an increase in pH and so less favourable conditions for bone resorption. Blood pH decreases from mid to late lay in this group however ionised blood calcium does not significantly increase possibly demonstrating the lack of mineral available within the bones at this time. Further the unselected strain (strain 2) demonstrated significantly higher blood ionised calcium and lower blood pH at late lay when compared to the selected strain. This supports the results of Bell & Siller (1962) who demonstrated reduced blood ionised calcium in birds experiencing osteoporosis. The levels of blood ionised calcium in the selected commercial line fell below those of the unselected J.Line at both early and late lay, whilst blood pH was higher at these times. The J.Line demonstrated a similar decrease in blood ionised calcium to mid lay but there was a significant increase to late lay accompanied by a favourable blood pH.

Gunaratne & Boorman (1996) found that the extent of plasma phosphorus mobilisation in the hen was representative of the amount of bone mobilisation taking place. In the current experiment the mean concentration of total plasma phosphorus decreased between early and mid lay in the selected commercial hybrid adding further weight to the results of the blood ionised calcium results, viz. that bone reserves are being used up in this

strain. In the unselected J.Line a decrease in mean total plasma phosphorus concentration is apparent from mid to late lay only when blood ionised calcium levels demonstrate a slight increase suggesting that there may be a decreased reliance on bone resorption at this time, possibly due to the decrease in production recorded.

The levels of total plasma magnesium remained constant in both strains as bone demands changed over lay thus adding weight to the argument proposed in Chapter 2 which stated that plasma magnesium was preferentially selected for incorporation into the eggshell at specific times during calcification rather than the eggshell being used as a dump for the excess plasma magnesium being produced by bone remodelling. It is hypothesised that the increased concentration of magnesium observed in the eggshell at mid lay is in response to a deficit expressed in the eggshell rather than to changes in bone remodelling *per se*. Such a deficit may correspond to the relationship already observed in the hatched egg between effective thickness and % eggshell magnesium. Bell & Siller (1962) demonstrated no difference in plasma magnesium concentration in birds presenting with osteoporosis.

Throughout lay the hens of the selected strain (strain 1) demonstrated significantly lower trabecular bone volume than the unselected J.Line confirming the results of Rennie *et al* (1997). Trabecular volume decreased minimally across lay in Strain 2. Although no statistically significant change in trabecular volume was apparent over lay in strain 1 a 29% decrease was recorded, this being similar to the 25 % decrease observed by Wilson *et al* (1992). Further, the levels observed in strain 1 fall largely below the 11% limit set by Wilson & Whitehead (1992) as being indicative of severe osteoporosis. Thus it is evident that even

from early in the lay period the commercial bird is demonstrating a predisposition to osteoporosis, as emphasised by Wilson *et al* (1992). This probably occurs due to the resorption of structural bone to supply medullary bone early in eggshell calcification, as a result of the high commercial egg output. Mueller *et al* (1964) demonstrated that in birds with a negative calcium balance more calcium was drawn into the medullary bone, thus decreasing the stability of the structural bone. Medullary bone volume increased in the selected strain between early and mid lay corresponding to the increase in egg weight at this time, whilst medullary bone volume only increased between mid and late lay in the unselected strain adding further weight to the argument that the stresses placed on the skeletal system of the selected strain (strain 1) were higher than those on the unselected strain (strain 2). Fleming *et al* (1996) demonstrated medullary bone contributing to overall strength in the humerus. As would be expected accompanying osteoporosis, bone density is significantly lower in the selected commercial strain, while the reproductive pattern of the largely unselected J.Line facilitates an increase in bone density across lay. Larger bones and a larger body weight combined with reduced structural bone, bone density and a high egg output means that the high incidence of osteoporosis was as expected in the highly productive commercial hybrid.

The % bone ash in the commercial hybrid starts below that of the unselected J.Line but increases between early and mid lay due to an increase in medullary bone volume. However no increase in calcium, phosphorus or magnesium are apparent thus as suggested earlier a further mineral constituent must be called into play at this time. Taylor & Moore (1956) demonstrated an increased absorption of sodium and potassium along with phosphorus and magnesium during times of calcium deprivation. It is possible that these minerals make up the differences

observed between % bone and eggshell ash and the minerals measured. The higher % phosphorus component of the J.Line bone at early lay may reflect the high trabecular volume at this time. Although there was no change in the mean % calcium at late lay, it is probable that the distribution of calcium within the bone had altered. Previous authors have demonstrated the poorly mineralised composition of structural bone in osteoporotic bone, whilst medullary bone remains unaffected (Bernard *et al*, 1980; Whitehead & Wilson, 1992; Wilson *et al*, 1992).

In conclusion, the relationship between eggshell quality and bone histomorphometry was evident in this multidisciplinary study. In the newly hatched egg, both lines demonstrated similar ultrastructural quality in terms of variants which would significantly impair calcium uptake from the eggshell. This was reflected in femur mineral compositions. Further, the J.Line strain which demonstrates poor hatchability in the field, had increased eggshell thickness due to ultrastructural variation at the mamillary level. Within the J.Line strain the element magnesium was found to be related to both eggshell thickness and eggshell resorption.

The adult commercial laying hen exhibited significantly higher production records and egg weight than the J.Line strain, thus placing a higher demand on the skeletal system, the effects of which were evident from the start of the experiment. This poor bone histomorphometry was accompanied by poorer ultrastructural shell quality at the beginning of lay. The level of osteoporotic compromise in the commercial strain was further reflected in % seconds and eggshell strength at the end of lay.

The novel assay for osteocalcin provided a useful tool to accompany bone histomorphometry. This assay demonstrated the

increased rate of bone remodelling at late lay in the commercial hybrid which corresponded to the expected increase in osteoid formation. Elevated osteocalcin concentrations were apparent in the J.Line reflecting the increased rate of osteoid formation necessary for the upkeep of structural bone in this strain. The assay for osteocalcin also demonstrated the changes in bone metabolism which occurred due to an increase in egg size in the J.Line strain. Although the assay for alkaline phosphatase showed no difference between the two lines throughout the period of lay, its incorporation into the experimental protocol was essential to demonstrate that although osteoid formation was altered this did not result in any change in bone mineralisation.

THE EFFECTS OF FEEDING A PRE-LAY DIET ON  
EGG-SHELL QUALITY AND ON THE BLOOD AND BONE  
BIOLOGY OF THE LAYING HEN



# THE EFFECTS OF FEEDING A PRE-LAY DIET ON EGGSHELL QUALITY AND ON THE BLOOD AND BONE BIOLOGY OF THE LAYING HEN.

## INTRODUCTION

### **CHAPTER 4**

## **THE EFFECTS OF FEEDING A PRE-LAY DIET ON EGGSHELL QUALITY AND ON THE BLOOD AND BONE BIOLOGY OF THE LAYING HEN.**

Medullary bone is formed in response to a combination of the action of oestrogen and parathyroid hormone (Taylor & Stringer, 1966). When sexual maturity is attained approximately 14-15 days prior to the onset of oviposition (Harris, 1964). Formation commences with the growth of the bony spicules which develop from the endosteal wall of the marrow bone. By first egg the spicules have developed into a dense network which Bloom et al (1968) observed to be the major site of resorption of the marrow cavity. These spicules have been demonstrated to house a large population of osteoclasts (Harris, 1964; Taylor & Stringer, 1965; Miller, 1971; Wainman et al, 1984). It is suggested in chapter 5 the histological pattern of medullary bone differs from that of cortical bone in that osteoclasts are hydroxyapatite is present in the A-band zone of the collagen fibre (Taylor et al, 1971). Whereas in cortical bone it is randomly distributed throughout



## **4 THE EFFECTS OF FEEDING A PRE-LAY DIET ON EGGSHELL QUALITY AND ON THE BLOOD AND BONE BIOLOGY OF THE LAYING HEN.**

### **4.1 INTRODUCTION**

The presence of medullary bone within the avian skeletal system (Taylor & Moore, 1954) has already been referred to in Chapters 1-3. Medullary bone was first observed by Kyes & Potter (1934) in the pigeon, where the extent of marrow ossification was related to follicular development. Medullary bone is morphologically adapted for its role of calcium provision during eggshell calcification (Miller, 1992), with a highly mineralised, large surface area in close connection to the vascular system, allowing calcium to be metabolised 10-15 times faster than in cortical bone (Hurwitz, 1965; Simkiss, 1967).

Medullary bone is formed, in response to a combination of the actions of androgen and oestrogen (Taylor & Stringer, 1965) during sexual maturation, approximately 14-16 days prior to the onset of egg production (Hurwitz, 1964). Formation commences with the growth of fine bony spicules which develop from the endosteal wall of the cortical bone. By first egg the spicules have developed into a dense meshwork which Bloom *et al* (1958) observed to fill the outer third or half of the marrow cavity. These spicules have been demonstrated to house a large population of osteoclasts (Bloom *et al*, 1958; Taylor & Stringer, 1965; Miller, 1992; Van de Velde *et al*, 1984). As suggested in chapter 3 the hydroxyapatite portion of medullary bone differs from that of structural bone. Within structural bone hydroxyapatite is present at the A-band level of the collagen fibrils (Taylor *et al*, 1971) whereas in medullary bone it is randomly distributed throughout

the tissue (Ascenzi *et al*, 1963). This leads to the woven structure of medullary bone which facilitates calcium withdrawal (Candlish, 1971b). It also permits the resorption of medullary bone when follicular activity ceases (Wilson *et al*, 1992) as in seasonal layers or brooder hens. However, in the modern commercial hybrid, medullary bone is present over the majority of the bird's life (Miller, 1992) and plays a crucial role in calcium dynamics, through the processes of bone resorption, osteoid formation and osteoid mineralisation (Van de Velde *et al*, 1985; Chapter 2).

The prelay diet is a relatively recent addition to the feed, only becoming standard practice in the last 10-15 years. The argument behind its introduction is as follows. It was hypothesised that by feeding a diet at the time corresponding to the onset of medullary bone formation, there would be an increased source of calcium available for its formation and thus benefits would be afforded at both early and late lay and in long term bone structure. Thus the nutritional gap was filled between the grower diet, designed to control the growth and development of the pullet, and the layer diet, which supplies a high calcium concentration for eggshell calcification. Immediately prior to lay the hen's appetite is largely calcium orientated and thus to feed the high calcium, layer diet at this time would lead to a reduction in dietary intake and thus a decrease in the absorption of the constituents necessary for muscle growth (Portsmouth, 1994).

The argument behind the prelay diet is fundamentally flawed. In 1964 Hurwitz reported that increased calcium uptake in the 14-16 days prior to the onset of egg production indeed reduced calcium depletion from the structural bone of the laying hen, but that this was due to its incorporation into the structural component of the bone. In other words, medullary bone was largely unaffected by calcium supplementation during its formation. This said, it is still

unclear if the provision of extra calcium supplementation contributes to long term bone morphology.

One of the few available studies into the provision of a prelay diet was carried out by a group at Harper Adams Agricultural College (1990), who demonstrated inconsistent changes in egg characteristics at early and late lay due to the feeding of either a prelay or layer diet at 16 weeks to ISA Brown hens. A further trial in 1991 which incorporated bone strength data also failed to produce conclusive results. Despite these inconsistencies and the lack of hard evidence, the layer manuals for Lohmann Brown, Hyline Brown, Hyline W-98, and Hyline W-36 still recommend that a prelay diet should be fed from 15 weeks to 5% lay, that is immediately after moving to intensive systems. The ISA Brown manual is slightly more flexible with prelay diets offered 2 weeks before the time when 2% lay is expected, which corresponds roughly to the period of medullary bone formation.

The aims of this chapter were to evaluate the effects of feeding a prelay diet, from 14-16 weeks of age, on all aspects of long term bird quality, that is productivity, eggshell quality and bone morphology. This study was primarily concerned with the vulnerable early and late lay periods (Chapter 3).

## **4.2 MATERIALS & METHODS**

### **4.2.1 PRODUCTION VARIABLES**

90 female commercial hybrid chicks were hatched and then housed in a brooder until four weeks of age before being transferred to individual bird cages. Standard management techniques were applied in all stages of development (Roslin Institute *pers comm*) (Appendix 3). Within the battery cage system birds were assigned to two groups. Care was taken to avoid cage position bias within and between the groups of birds, thus equal numbers of birds were placed randomly in both tiers of the system. Group 1 birds were fed on standard rations throughout the study period (i.e. starter diet, pre-lay diet, layer diet), Group 2 birds were maintained on a starter diet until lay commenced, then fed a layer diet thereafter, thus omitting the pre-lay diet stage. A full dietary analysis can be seen in Appendices 1 and 2. From 5% lay (approximately 18 weeks) production records were kept. These detailed the number of eggs laid per hen per week and the % seconds per hen per week (those eggs which would be routinely graded out on farm) (Appendix 4). Egg weight was recorded during the experimental periods (g/hen/day).

### **4.2.2 GENERAL EXPERIMENTATION**

At 25, 45 and 67 weeks of age, 15 birds (where possible) were selected at random from each group and treated as follows: 14 consecutive eggs per bird were collected, pooled on a group basis and subjected to ultrastructural assessment (3.2.5). The eggs collected from the fifteenth day were assessed using traditional measures (3.2.6), material properties (3.2.7) and

mineral composition (3.2.8) procedures. On the 19th day, the birds were blood sampled then sacrificed for the assessment of bone histomorphometry (3.2.4). Blood sampling and sacrifice took place at approximately peak eggshell mineralisation (10 hours post-oviposition) which was determined by manual recording of the previous egg laid. Those birds without a calcifying egg in the shell gland pouch were discarded from the experiment. Data analysis was carried out as described in 3.2.9.

### 4.1.1 PRODUCTION VARIABLES

In order to assess differences in the egg weight and % shell weight between egg and in assessing the effect of treatment on egg weight and % shell weight, the data were analysed using a two-way ANOVA. The data were then used to calculate the corresponding egg weight and % shell weight for each group (early and late) and the data were analysed using a two-way ANOVA.

Production (Figure 23) was maintained at similar levels in both groups and decreased with bird age. The % shell weight (Figure 24) was significantly higher in Group 2. The data carried out in this experiment were analysed using a two-way ANOVA. The data were then used to calculate the corresponding egg weight and % shell weight for each group (early and late) and the data were analysed using a two-way ANOVA. The data were then used to calculate the corresponding egg weight and % shell weight for each group (early and late) and the data were analysed using a two-way ANOVA. The data were then used to calculate the corresponding egg weight and % shell weight for each group (early and late) and the data were analysed using a two-way ANOVA.



### **4.3 RESULTS**

The means and standard deviations of the means corresponding to early, mid and late lay in each group are displayed for all variables in Tables 12-17. The results of the general linear model (glm) for group|age interactions associated with each variable are also presented. All significant data are represented in Figures 63-91, accompanied by standard deviation of the mean.

#### **4.3.1 PRODUCTION VARIABLES**

In order to assess differences in the egg number, weight and % seconds of eggs per hen per day the three weeks data collected over the experimental period was expressed as a mean value per hen. These data were then used to calculate the corresponding mean and standard errors for each group at early, mid and late lay (Table 12).

Production (Figure 63) was maintained at similar levels in both groups and decreased with bird age. The % seconds (Figure 64) recorded were significantly higher in Group 1. The glm carried out on this parameter recorded no age effects in this group however due to the extreme high value of % seconds a post-hoc t-test was carried out which demonstrated a significant increase in % seconds at late lay. In Group 2 % seconds remained constant across the laying period. Egg weight (g/hen/day) (Figure 65) increased in both groups between early and mid lay, then remained constant between mid and late lay.

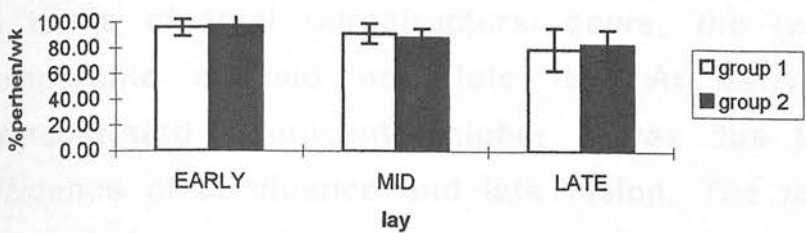


VARIABLE	PRODUCTION (%/hen/wk)		EGG WEIGHT (g/hen/day)		SECONDS (%/hen/wk)	
	1	2	1	2	1	2
GROUP						
EARLY (st.dev)	95.6 <sup>a</sup> (6.58)	97.07 <sup>a</sup> (8.37)	1.46 <sup>a</sup> (3.00)	3.98 <sup>a</sup> (4.19)	61.24 <sup>a</sup> (3.54)	59.45 <sup>a</sup> (2.14)
MID (st.dev)	91.21 <sup>ac</sup> (7.74)	88.1 <sup>bc</sup> (7.18)	0.88 <sup>ab</sup> (2.18)	2.87 <sup>a</sup> (5.09)	70.44 <sup>b</sup> (3.93)	68.61 <sup>ab</sup> (3.11)
LATE (st.dev)	79.59 <sup>b</sup> (16.43)	83.07 <sup>b</sup> (11.93)	14.21 <sup>b</sup> (27.50)	5.25 <sup>a</sup> (8.61)	67.89 <sup>b</sup> (13.03)	70.52 <sup>b</sup> (5.54)
Group	n s	n s	n s	n s	n s	n s
Age	**	**	*	*	**	**
Group/Age	n s	n s	n s	n s	n s	n s

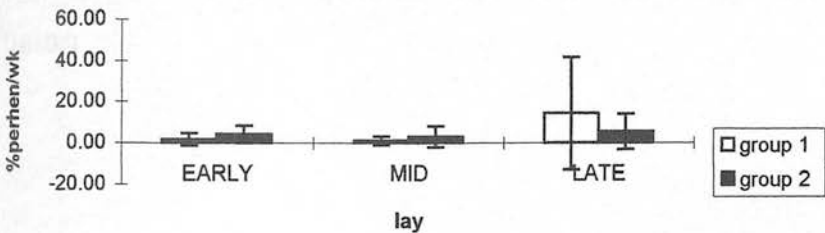
**Table 12:** Influence of group and age on production variables in the laying hen.

(st.dev) = standard deviation of the mean; a-c = means with same superscript do not differ significantly ( $p < 0.05$ );

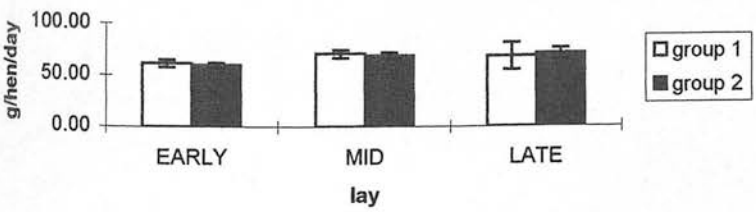
\*\*  $p < 0.01$ ; \*  $p < 0.05$ ; ns not statistically significant.



**Figure 63:** Mean production over lay incorporating standard error of the mean.



**Figure 64:** Mean % seconds over lay incorporating standard error of the mean.



**Figure 65:** Mean egg weight over lay incorporating standard error of the mean.

### **4.3.2 ULTRASTRUCTURAL MEASURES**

In terms of total ultrastructural score, the two groups were comparable at mid and late lay. At early lay Group 1 demonstrated significantly higher scores due to an increased incidence of confluence and late fusion. The total score value decreased across lay in Group 1 and between mid and late lay in Group 2. The specific structural variants making up the total score varied both between and within groups with increasing age (Table 13; Figure 66-72).

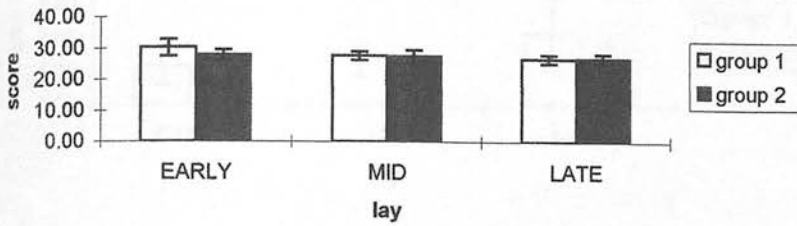
Neither total nor effective thickness varied between or within groups across lay. The mammillary thickness in Group 2 (Figure 73) was however significantly lower at early lay indicating early fusion.

VARIABLE	CONFLUENCE		FUSION		CUFFING		ALIGNMENT		TYPE B		DEPRESSION		EROSION		CUBIC		ARAGONITE	
	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
GROUP																		
EARLY (st.dev)	3.15 <sup>a</sup> (0.38)	2.00 <sup>b</sup> (0.82)	7.62 <sup>a</sup> (1.04)	6.62 <sup>d</sup> (0.87)	7.00 (0.00)	7.00 (0.00)	3.00 (0.00)	3.00 (0.00)	3.15 <sup>a</sup> (0.38)	3.31 <sup>a</sup> (0.48)	1.00 (0.00)	1.08 (0.28)	1.15 <sup>a</sup> (0.55)	1.00 <sup>a</sup> (0.00)	2.46 <sup>a</sup> (0.88)	2.08 <sup>a</sup> (0.49)	1.54 (1.13)	1.00 (0.00)
MID (st.dev)	1.92 <sup>b</sup> (0.76)	1.82 <sup>bc</sup> (0.60)	7.54 <sup>b</sup> (0.88)	7.73 <sup>b</sup> (0.79)	7.00 (0.00)	7.00 (0.00)	3.15 (0.55)	3.18 (0.75)	3.39 <sup>ab</sup> (0.51)	3.64 <sup>a</sup> (0.81)	1.00 (0.00)	1.00 (0.00)	1.39 <sup>a</sup> (0.51)	1.18 <sup>ac</sup> (0.40)	1.23 <sup>b</sup> (0.60)	1.27 <sup>b</sup> (0.47)	1.08 (0.28)	1.36 (0.92)
LATE (st.dev)	1.00 <sup>c</sup> (0.00)	1.33 <sup>c</sup> (0.50)	4.71 <sup>c</sup> (0.95)	5.22 <sup>c</sup> (0.83)	7.00 (0.00)	7.00 (0.00)	3.14 (0.38)	3.00 (0.00)	3.86 <sup>b</sup> (1.07)	3.11 <sup>a</sup> (0.33)	1.14 (0.38)	1.00 (0.00)	2.14 <sup>b</sup> (1.07)	1.33 <sup>c</sup> (0.50)	1.00 <sup>b</sup> (0.00)	1.00 <sup>b</sup> (0.00)	1.14 (0.38)	1.00 (0.00)
Group	*		**		ns		ns		ns		ns		**		ns		ns	
Age	**		ns		ns		ns		ns		ns		**		**		ns	
Group Age	**		*		ns		ns		*		ns		ns		ns		ns	

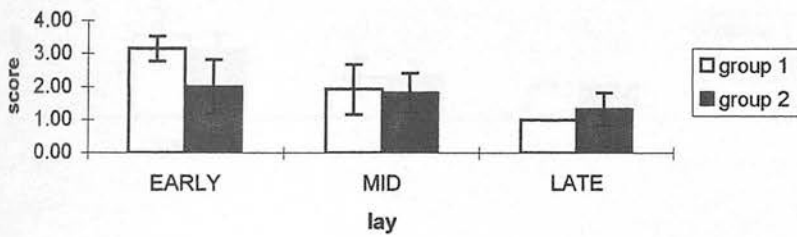
**Table 13:** Influence of group and age on ultrastructural eggshell quality variables in the laying hen. (st. dev) = standard deviation of the mean; a-c = means with same superscript do not differ significantly ( $p < 0.05$ ); \*\*  $p < 0.01$ ; \*  $p < 0.05$ ; ns not statistically significant.

VARIABLE	CAPS		TYPE A		CHANGED MEMBRANE		TOTAL THICKNESS (mm)		MAMMILLARY THICKNESS (mm)		EFFECTIVE THICKNESS (mm)	
	1	2	1	2	1	2	1	2	1	2	1	2
GROUP												
EARLY (st.dev)	4.46 <sup>a</sup> (0.78)	4.31 <sup>a</sup> (0.63)	1.00 (0.00)	1.00 (0.00)	1.92 <sup>a</sup> (0.28)	2.08 <sup>a</sup> (0.64)	0.309 (0.02)	0.296 (0.01)	0.055 <sup>a</sup> (0.01)	0.040 <sup>b</sup> (0.01)	0.258 (0.02)	0.256 (0.01)
MID (st.dev)	4.77 <sup>ac</sup> (0.60)	5.00 <sup>c</sup> (0.45)	1.00 (0.00)	1.00 (0.00)	1.39 <sup>b</sup> (0.51)	1.36 <sup>b</sup> (0.50)	0.305 (0.02)	0.306 (0.02)	0.047 <sup>a</sup> (0.01)	0.049 <sup>ab</sup> (0.01)	0.257 (0.02)	0.257 (0.02)
LATE (st.dev)	3.57 <sup>b</sup> (0.79)	4.67 <sup>ac</sup> (0.71)	1.00 (0.00)	1.00 (0.00)	1.71 <sup>ab</sup> (0.76)	1.44 <sup>b</sup> (0.53)	0.310 (0.02)	0.318 (0.03)	0.050 <sup>a</sup> (0.01)	0.061 <sup>a</sup> (0.02)	0.260 (0.01)	0.258 (0.04)
Group	*		ns		ns		ns		ns		ns	
Age	**		ns		**		ns		**		ns	
Group(Age)	*		ns		ns		ns		ns		ns	

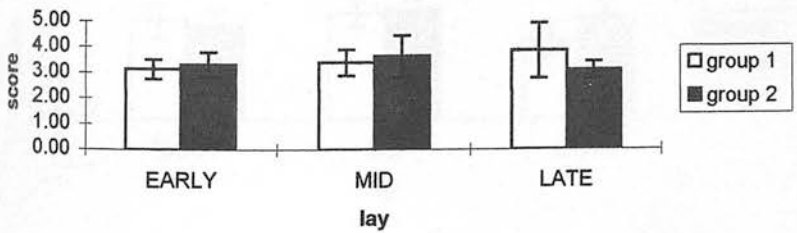
**Table 13 (continued):** Influence of group and age on ultrastructural eggshell quality variables in the laying hen. (st. dev) = standard deviation of the mean; a-c = means with same superscript do not differ significantly ( $p < 0.05$ ); \*\*  $p < 0.01$ ; \*  $p < 0.05$ ; ns not statistically significant.



**Figure 66:** Mean total ultrastructural score over lay incorporating standard error of the mean.

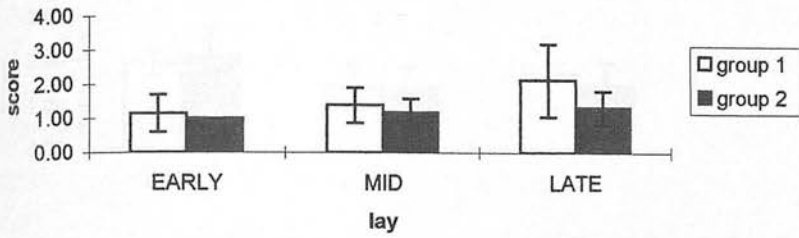


**Figure 67:** Mean confluence score over lay incorporating standard error of the mean.

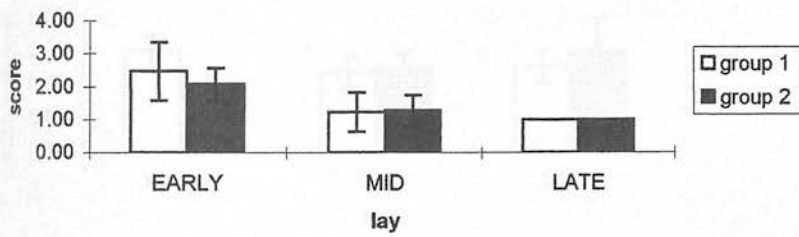


**Figure 68:** Mean type B score over lay incorporating standard error of the mean.

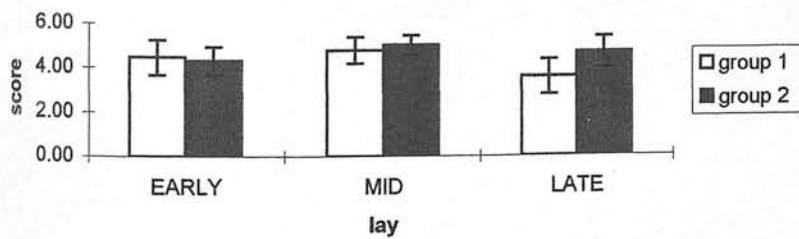




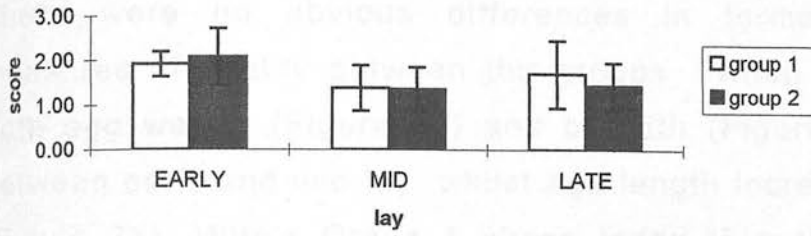
**Figure 69:** Mean erosion score over lay incorporating standard error of the mean.



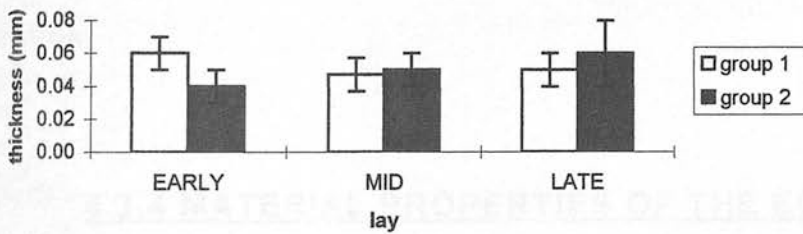
**Figure 70:** Mean cubic score over lay incorporating standard error of the mean.



**Figure 71:** Mean cap score over lay incorporating standard error of the mean.



**Figure 72:** Mean changed membrane over lay incorporating standard error of the mean.



**Figure 73:** Mean mammillary thickness over lay incorporating standard error of the mean.

### **4.3.3 TRADITIONAL MEASURES**

There were no obvious differences in terms of traditional measures of quality between the groups. Within the two groups both egg weight (Figure 74) and breadth (Figure 76) increased between early and mid lay, whilst egg length increased across lay (Figure 75). Within Group 1 shape index (Figure 77) increased between mid and late lay, whilst strength (Figure 79) decreased and deformation (Figure 78) remained constant. Shape index increased between early and mid lay in group 2, while deformation increased across lay and strength decreased across lay. The measure of stiffness remained constant with age in both groups.

### **4.3.4 MATERIAL PROPERTIES OF THE EGGSHELL**

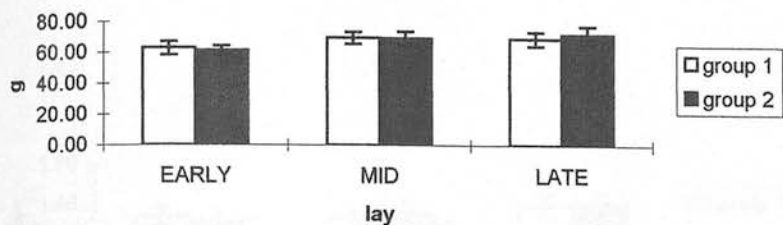
Both elastic modulus and fracture toughness were comparable between groups. Elastic modulus remained constant within each group across lay. In group 1, fracture toughness (Figure 80) demonstrated a significant decrease between mid and late lay whilst in group 2, it decreased throughout the entire study period.

VARIABLE	EGG WEIGHT (g)		EGG LENGTH (mm)		EGG BREADTH (mm)		SHAPE INDEX		DEFORMATION ( $\mu$ m)		STRENGTH (N)		STIFFNESS (N/mm)		ELASTIC MODULUS (Nm m-2)		FRACTURE TOUGHNESS (Nm m-3/2)	
GROUP	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
EARLY (st.dev)	62.80 <sup>a</sup> (4.38)	61.18 <sup>a</sup> (3.06)	57.7 <sup>a</sup> (1.73)	56.7 <sup>a</sup> (1.64)	43.6 <sup>a</sup> (1.13)	43.3 <sup>a</sup> (1.11)	1.33 <sup>a</sup> (0.05)	1.31 <sup>a</sup> (0.05)	19.0 <sup>a</sup> (3.20)	17.6 <sup>a</sup> (1.90)	30.7 <sup>a</sup> (5.63)	33.5 <sup>a</sup> (6.49)	189 (34.36)	200 (21.29)	35883 (6610)	37592 (5847)	608 <sup>a</sup> (154.09)	660 <sup>a</sup> (142.96)
MID (st.dev)	69.40 <sup>b</sup> (3.90)	69.14 <sup>b</sup> (4.38)	59.9 <sup>b</sup> (2.38)	61.0 <sup>b</sup> (2.26)	45.0 <sup>b</sup> (1.00)	44.8 <sup>b</sup> (1.35)	1.33 <sup>ac</sup> (0.03)	1.36 <sup>bc</sup> (0.06)	21.1 <sup>a</sup> (3.06)	19.9 <sup>ac</sup> (2.85)	31.4 <sup>a</sup> (2.73)	30.2 <sup>ab</sup> (5.52)	195 (31.96)	198 (35.01)	38121 (6281)	37973 (11269)	609 <sup>a</sup> (83.07)	592 <sup>ac</sup> (122.44)
LATE (st.dev)	69.20 <sup>b</sup> (4.64)	72.01 <sup>b</sup> (5.69)	62.1 <sup>c</sup> (2.63)	63.4 <sup>cd</sup> (1.87)	44.7 <sup>ab</sup> (1.03)	44.9 <sup>b</sup> (1.63)	1.39 <sup>b</sup> (0.06)	1.41 <sup>b</sup> (0.05)	22.1 <sup>ac</sup> (2.60)	23.0 <sup>c</sup> (3.72)	22.4 <sup>b</sup> (4.45)	23.5 <sup>b</sup> (5.56)	182 (36.46)	163 (29.67)	34750 (4778)	34849 (13870)	420 <sup>b</sup> (63.90)	461 <sup>bc</sup> (95.96)
Group	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Age	**	**	**	**	**	**	**	**	**	**	**	**	ns	ns	ns	ns	**	**
Group Age	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

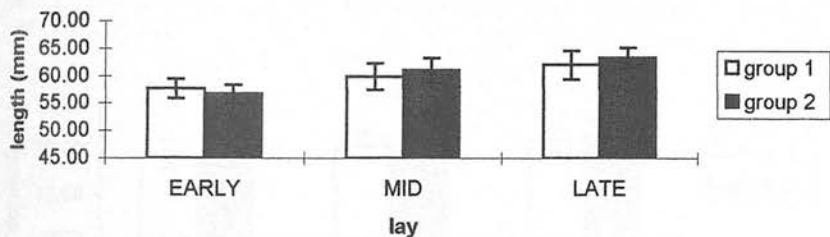
**Table 14:** Influence of group and age on egg variables in the laying hen.

(st. dev) = standard deviation of the mean; a-c = means with same superscript do not differ significantly ( $p < 0.05$ );

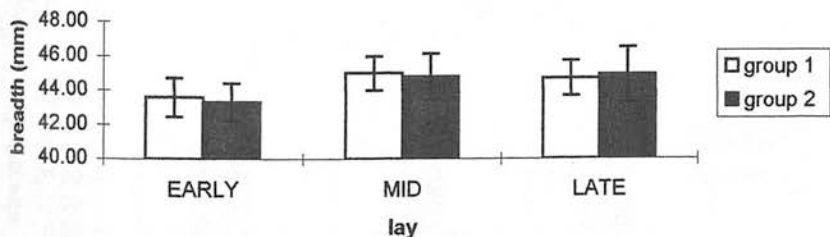
\*\*  $p < 0.01$ ; \*  $p < 0.05$ ; ns not statistically significant.



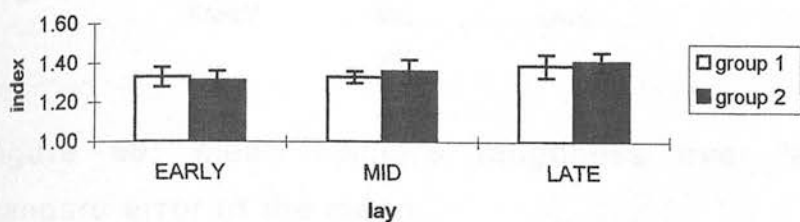
**Figure 74:** Mean egg weight over lay incorporating standard error of the mean.



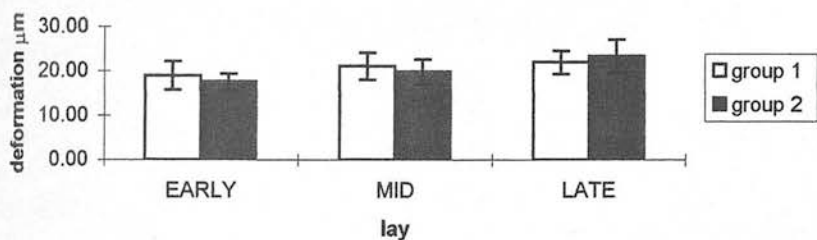
**Figure 75:** Mean egg length over lay incorporating standard error of the mean.



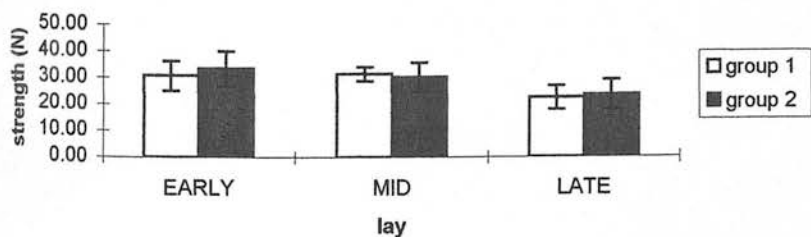
**Figure 76:** Mean egg breadth over lay incorporating standard error of the mean.



**Figure 77:** Mean shape index over lay incorporating standard error of the mean.

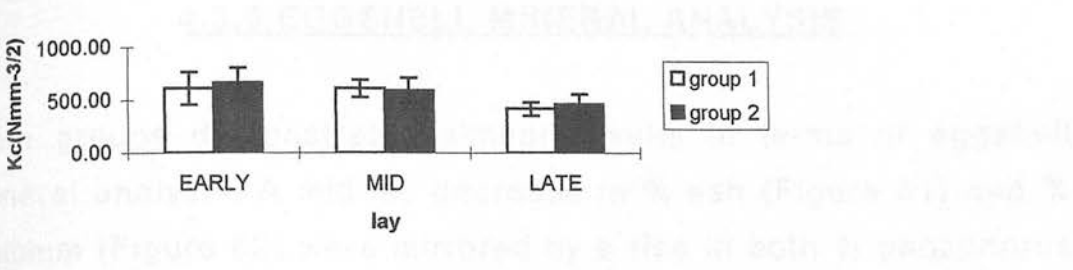


**Figure 78:** Mean deformation over lay incorporating standard error of the mean.



**Figure 79:** Mean strength over lay incorporating standard error of the mean.





**Figure 80:** Mean fracture toughness over lay incorporating standard error of the mean.

#### 4.3.5 EGGSHELL MINERAL ANALYSIS

Both groups demonstrated similar results in terms of eggshell mineral analysis. A mid lay decrease in % ash (Figure 81) and % calcium (Figure 82) were mirrored by a rise in both % phosphorus (Figure 83) and magnesium (Figure 84).

VARIABLE	%ASH (dry fat free)		% CALCIUM		% PHOSPHORUS		% MAGNESIUM	
	1	2	1	2	1	2	1	2
GROUP								
EARLY	95.2 <sup>a</sup>	93.18 <sup>a</sup>	44.97 <sup>a</sup>	50.06 <sup>a</sup>	0.055 <sup>a</sup>	0.058 <sup>a</sup>	0.590 <sup>a</sup>	0.453 <sup>a</sup>
(st.dev)	(2.52)	(3.24)	(9.27)	(14.04)	(0.03)	(0.03)	(0.18)	(0.17)
MID	84.17 <sup>b</sup>	86.49 <sup>b</sup>	34.15 <sup>b</sup>	34.64 <sup>b</sup>	0.308 <sup>b</sup>	0.294 <sup>b</sup>	3.726 <sup>b</sup>	3.951 <sup>b</sup>
(st.dev)	(8.87)	(9.70)	(2.28)	(1.58)	(0.13)	(0.09)	(0.50)	(1.01)
LATE	94.66 <sup>a</sup>	97.16 <sup>a</sup>	32.71 <sup>b</sup>	34.18 <sup>b</sup>	0.068 <sup>a</sup>	0.062 <sup>a</sup>	0.495 <sup>a</sup>	0.587 <sup>a</sup>
(st.dev)	(4.96)	(1.07)	(4.83)	(6.54)	(0.02)	(0.03)	(0.09)	(0.12)
Group	n s		n s		n s		n s	
Age	**		**		**		**	
Group/Age	n s		n s		n s		n s	

**Table 15:** Influence of group and age on eggshell mineral in the laying hen.

(st. dev) = standard deviation of the mean; a-b = means with same superscript do not differ significantly ( $p < 0.05$ );

\*\*  $p < 0.01$ ; \*  $p < 0.05$ ; ns not statistically significant.

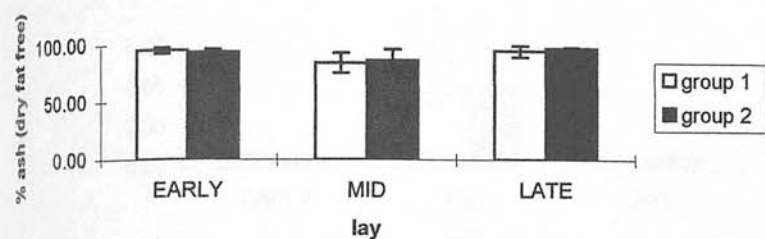


Figure 81: Mean eggshell % ash over lay incorporating standard error of the mean.

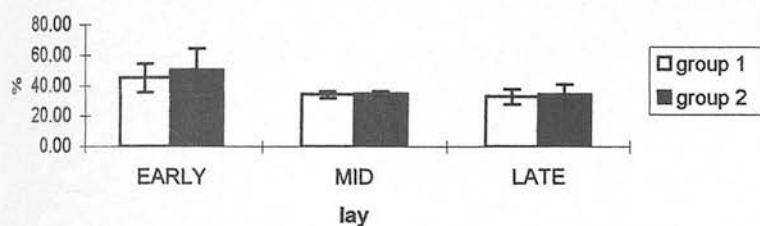


Figure 82: Mean eggshell % calcium over lay incorporating standard error of the mean.

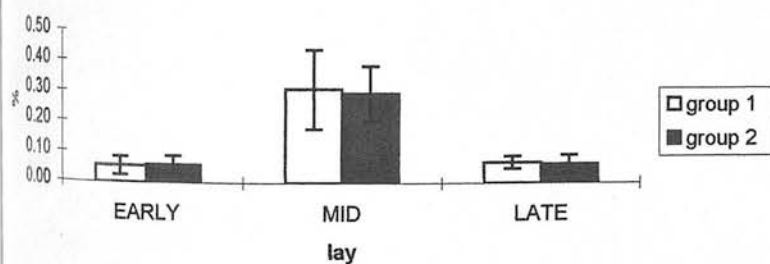
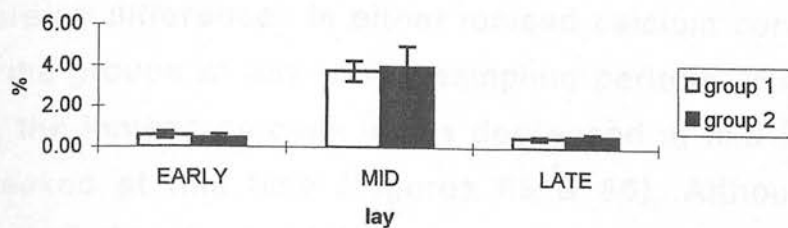


Figure 83: Mean eggshell % phosphorus over lay incorporating standard error of the mean.



**Figure 84:** Mean eggshell % magnesium over lay incorporating standard error of the mean.

#### **4.3.6 BLOOD ANALYSES**

There were no differences in either ionised calcium concentration or pH between the groups at any of the sampling periods. Within both groups however, the ionised calcium levels decreased at mid lay whilst the pH values peaked at this time (Figures 85 & 86). Although the Group 1 concentration of ionised calcium at late lay remained lower than that at early lay it was not significantly different to that displayed by Group 2 at this time.

Total plasma calcium levels (Figure 87) were greater in Group 2 at mid and late lay whilst plasma magnesium, phosphorus (Figure 88) and alkaline phosphatase concentrations were comparable between groups throughout lay. The levels of plasma phosphorus decreased between early and mid lay in Group 1. Within Group 2 differences in phosphorus levels were less obvious.

Osteocalcin concentration increased across lay in both groups, however in Group 1 the increase occurred between mid and late lay, whilst in group 2 concentrations increased continuously across lay (Figure 89).

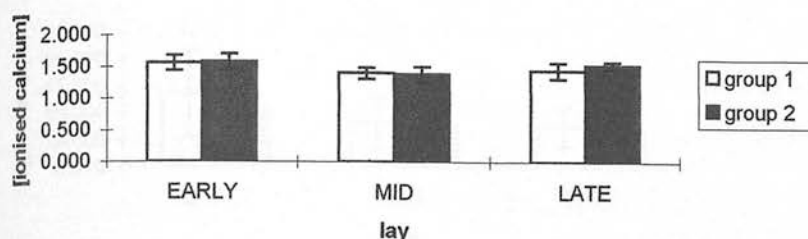


VARIABLE	IONISED CALCIUM		pH		TOTAL CALCIUM		TOTAL PHOSPHORUS		TOTAL MAGNESIUM		ALKALINE PHOSPHATASE		OSTEOCALCIN	
	1	2	1	2	1	2	1	2	1	2	1	2	1	2
EARLY (st.dev)	1.56 <sup>a</sup> (0.12)	1.57 <sup>a</sup> (0.13)	7.38 <sup>a</sup> (0.06)	7.36 <sup>a</sup> (0.05)	5.72 <sup>a</sup> (0.92)	6.71 <sup>ab</sup> (1.30)	13.73 <sup>a</sup> (8.17)	10.58 <sup>a</sup> (2.91)	3.72 (3.74)	2.75 (1.29)	13.48 (4.96)	16.38 (5.79)	3.8 <sup>a</sup> (3.82)	6.24 <sup>a</sup> (3.60)
MID (st.dev)	1.4 <sup>b</sup> (0.09)	1.38 <sup>b</sup> (0.12)	7.5 <sup>b</sup> (0.07)	7.49 <sup>b</sup> (0.04)	5.54 <sup>a</sup> (1.70)	6.53 <sup>b</sup> (1.07)	8.4 <sup>b</sup> (4.78)	8.25 <sup>ab</sup> (6.25)	1.62 (0.45)	1.66 (0.41)	13.59 (5.99)	12.45 (6.51)	7.66 <sup>a</sup> (6.96)	14.3 <sup>ab</sup> (5.32)
LATE (st.dev)	1.44 <sup>bc</sup> (0.13)	1.53 <sup>ac</sup> (0.06)	7.41 <sup>a</sup> (0.04)	7.38 <sup>a</sup> (0.04)	6.45 <sup>a</sup> (2.62)	7.10 <sup>b</sup> (1.85)	7.7 <sup>bc</sup> (3.66)	6.89 <sup>ac</sup> (2.42)	2.98 (2.62)	5.19 (8.37)	13.44 (4.23)	8.20 (4.34)	20.4 <sup>b</sup> (11.87)	25.68 <sup>b</sup> (12.67)
Group	ns		ns		*		ns		ns		ns		ns	
Age	**		**		ns		**		ns		ns		**	
Group/Age	ns		ns		ns		ns		ns		ns		ns	

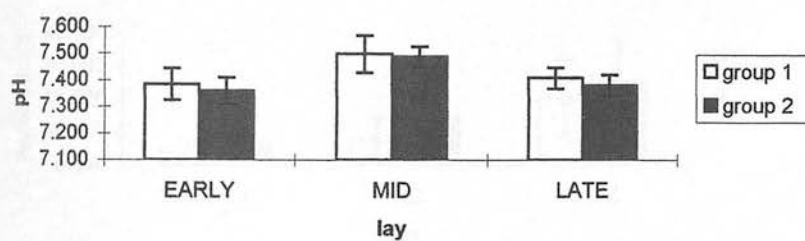
**Table 16:** Influence of group and age on blood parameters in the laying hen.

(st. dev) = standard deviation of the mean; a-c = means with same superscript do not differ significantly ( $p < 0.05$ );

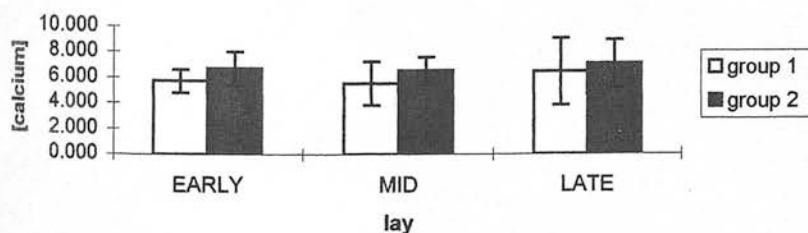
\*\*  $p < 0.01$ ; \*  $p < 0.05$ ; ns not statistically significant.



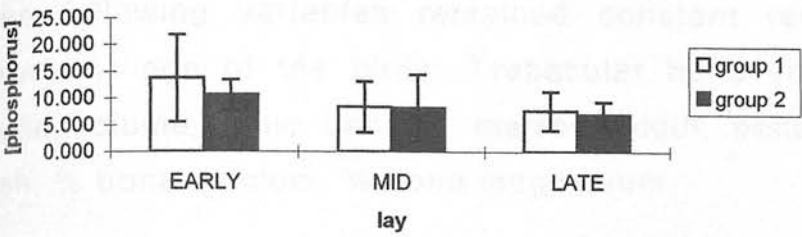
**Figure 85:** Mean ionised blood calcium concentration over lay incorporating standard error of the mean.



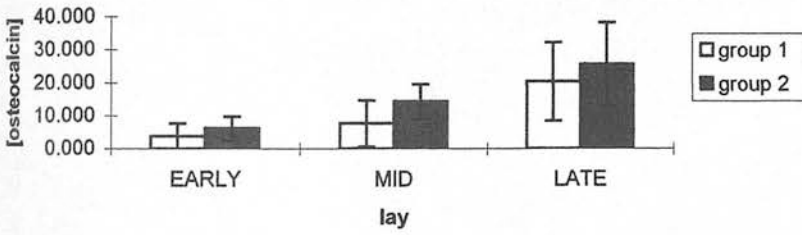
**Figure 86:** Mean blood pH over lay incorporating standard error of the mean.



**Figure 87:** Mean total plasma calcium concentration over lay incorporating standard error of the mean.



**Figure 88:** Mean plasma phosphorus concentration over lay incorporating standard error of the mean.



**Figure 89:** Mean plasma osteocalcin concentration over lay incorporating standard error of the mean.

#### **4.3.7 BONE HISTOMORPHOMETRY**

The following variables remained constant regardless of the group or age of the birds: Trabecular bone volume, medullary bone volume, bone density, marrow width, bone width, % bone ash, % bone calcium, % bone magnesium.

The K value (Figure 90) was significantly higher in Group 2 at late lay. % phosphorus (Figure 91) was comparable in both groups but varied with bird age, viz. % phosphorus levels increased from early to mid lay.

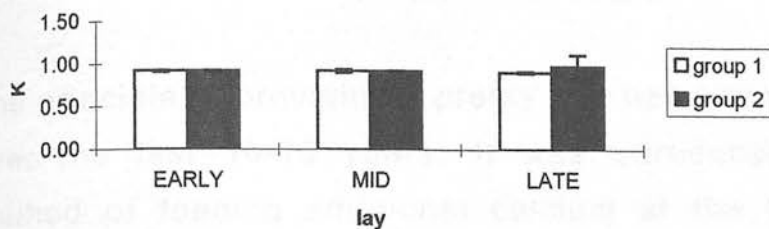
VARIABLE	TRABECULAR BONE VOLUME		MEDULLARY BONE VOLUME		BONE DENSITY		MARROW WIDTH (mm)		BONE WIDTH (mm)		K	
	1	2	1	2	1	2	1	2	1	2	1	2
GROUP												
EARLY (st.dev)	10.89 (3.34)	12.48 (3.10)	11.31 (1.71)	13.00 (7.08)	1.19 (0.16)	1.25 (0.15)	7.63 (0.25)	7.83 (0.37)	8.30 (0.25)	8.48 (0.37)	0.92 <sup>a</sup> (0.01)	0.92 <sup>ab</sup> (0.01)
MID (st.dev)	11.42 (4.85)	13.02 (6.38)	15.06 (4.95)	11.02 (4.75)	1.14 (0.14)	1.16 (0.23)	7.75 (0.33)	7.89 (0.47)	8.46 (0.29)	8.56 (0.49)	0.92 <sup>a</sup> (0.02)	0.92 <sup>ab</sup> (0.01)
LATE (st.dev)	7.66 (4.07)	11.51 (5.20)	17.45 (8.86)	8.04 (8.03)	1.14 (0.18)	1.22 (0.27)	7.81 (0.40)	7.83 (0.47)	8.54 (0.30)	8.06 (0.81)	0.91 <sup>a</sup> (0.01)	0.98 <sup>b</sup> (0.14)
Group	ns		ns		ns		ns		ns		*	
Age	ns		ns		ns		ns		ns		ns	
Group/Age	ns		ns		ns		ns		ns		ns	

**Table 17:** Influence of group and age on bone parameters in the laying hen. (st. dev) = standard deviation of the mean; a-c = means with same superscript do not differ significantly ( $p < 0.05$ ); \*\*  $p < 0.01$ ; \*  $p < 0.05$ ; ns not statistically significant.

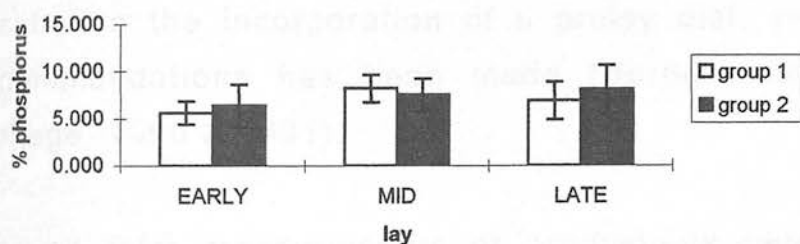
VARIABLE	%ASH (dry fat free)		% CALCIUM		% PHOSPHORUS		% MAGNESIUM	
	1	2	1	2	1	2	1	2
GROUP								
EARLY (st.dev)	56.88 (4.46)	58.38 (3.21)	21.84 (5.68)	26.29 (9.55)	5.64 <sup>a</sup> (1.21)	6.38 <sup>a</sup> (2.24)	0.52 (0.11)	0.60 (0.24)
MID (st.dev)	59.06 (2.22)	61.19 (3.10)	26.24 (10.99)	24.16 (11.55)	8.23 <sup>bc</sup> (1.45)	7.55 <sup>ac</sup> (1.75)	0.55 (0.20)	0.66 (0.37)
LATE (st.dev)	60.90 (5.56)	57.86 (6.79)	22.62 (8.71)	21.02 (20.27)	6.95 <sup>ba</sup> (2.00)	8.14 <sup>a</sup> (2.59)	0.53 (0.19)	0.53 (0.51)
Group	n s	n s	n s	n s	n s	n s	n s	n s
Age	n s	n s	n s	n s	**	**	n s	n s
Group Age	n s	n s	n s	n s	n s	n s	n s	n s

**Table 17 (continued):** Influence of group and age on bone parameters in the laying hen. (st. dev) = standard deviation of the mean; a-c = means with same superscript do not differ significantly ( $p < 0.05$ ); \*\*  $p < 0.01$ ; \*  $p < 0.05$ ; ns not statistically significant.





**Figure 90:** Mean bone K value over lay incorporating standard error of the mean.



**Figure 91:** Mean bone % phosphorus over lay incorporating standard error of the mean.

#### 4.4 DISCUSSION

The principle of providing a prelay diet has been common practice over the last 10-15 years. It was introduced as a balanced method of feeding additional calcium at the time of medullary bone formation, approximately 14-16 days prior to the onset of production (Hurwitz, 1964), thus in theory benefiting the early and late lay problem periods. However Hurwitz himself demonstrated that medullary bone composition was largely unaffected by calcium supplementation during its formation, although such supplementation may increase the calcium content of structural bone. Other studies have also failed to demonstrate conclusive results on the incorporation of a prelay diet, yet no change in recommendations has been made (Harper Adams Agricultural College, 1990 & 1991).

The on farm measurements of productivity generally take into account parameters such as those given in Table 12 and Figures 63-65. In many cases these would then be used to determine the efficacy or otherwise, of a treatment. From the data presented herein, it would initially appear that the provision of a prelay diet formulation has had no obvious effect on either egg production or weight (g/hen/day) *per se*. A more critical evaluation of the production data however reveals that the prelay diet has effectively enabled production to be maintained at a peak for longer and for higher egg weights to be achieved earlier. The benefits of this however, are questionable when considered in the light of the high % seconds at the end of lay in the prelay diet group. The high variability accompanying this result would appear to suggest that a few birds were producing high numbers of seconds and were possibly demonstrating a stress response. Watt (1985) demonstrated the high variation in response to stress by individual birds.

The process of eggshell mineralisation, reflecting as it does the interaction between the organic and inorganic components of the egg, throws up a variety of structural aberrations, some of which are detrimental to quality and others which assist in shell function (Reid, 1984). Eggshell quality in its broadest sense in the conventionally fed commercial hybrid, represented by group 1, improved significantly across lay which again is in marked contrast to the results of previous authors (Boorman *et al*, 1985; Watt, 1989; Bain, 1990; Roberts & Brackpool, 1993-4). As discussed in Chapter 3 the reason for this is likely to be due to the experimental environment employed in setting up this experiment.

In birds not fed a prelay diet (Group 2) eggshell quality was significantly better at early lay, due to a decrease in the incidence of confluence and late fusion. Although no significant difference between the strains was evident in terms of effective thickness, the eggs of the birds deprived of a prelay diet did demonstrate a significantly lower mammillary thickness at the beginning of lay.

At late lay, there was no age or strain related difference in total ultrastructural score. The eggs of those birds fed a prelay diet did however demonstrate significantly higher inclusions of both type B's and erosions, whilst those deprived of a prelay diet showed a higher incidence of poor cap quality. Given the relatively minor nature of these differences it is not possible to comment on whether the provision or lack of a prelay diet had initiated them. Under commercial conditions the incidence of type B's and erosion, with their inherent lack of bonding with membrane fibres predisposes shells to cracking. It should be noted that these variants were more evident in the prelay diet group at the end of lay, at which time they also displayed an apparently higher incidence of seconds.

With reference to the traditional measures of eggshell quality, the eggs studied in both groups were comparable in most respects. The measure of deformation demonstrated an increase at late lay in those birds deprived of a prelay diet. When considering the measures of elastic modulus and fracture toughness the values were similar between groups although the decrease observed at the end of lay commenced earlier in the group deprived of a prelay diet.

The pattern of age related eggshell mineral compositional changes discussed in chapter 3 was not altered by the withholding of the prelay diet. Both groups in this study demonstrated a decrease in eggshell ash and calcium at mid lay accompanied by an increase in both % phosphorus and magnesium. At late lay however, although % ash increased significantly this was achieved without any contribution by eggshell calcium which remained largely constant while % phosphorus and magnesium had both decreased. Although there were no significant differences between the two groups in terms of percentage mineral composition, the fact that the prelay diet group had a higher rate of egg production for longer and laid heavier eggs earlier will have led to a greater demand on mineral reserves.

Chapter 2 concluded that osteocalcin was a marker of the osteoid formation process occurring during eggshell calcification. Under normal circumstance bone resorption accompanies this event. Bone mineralisation is minimal during eggshell calcification (Van de Velde *et al*, 1985). In chapter 2 it was shown that total calcium decreases whilst inorganic phosphate increases due to bone resorption. Chapter 3 concentrated on blood related changes corresponding to mid eggshell calcification, when it was revealed

that, osteoid formation demonstrated an increase at late lay which was consistent with the large scale medullary bone remodelling occurring at this time. Further bone mineralisation, as evidenced by alkaline phosphatase activity, remained constant. From the sustained low levels of ionised calcium from mid lay despite optimal pH conditions and the accompanying decrease in total phosphorus concentration it was suggested that regardless of the obvious calcium demand, as evidenced by total calcium concentration, bone resorption was occurring at a lower rate pointing to a deficit occurring within the skeletal system.

The increase in osteoid formation appeared to commence earlier in the group without the prelay diet (Group 2), with no change in bone mineralisation, thus this group had more unmineralised bone at mid lay when compared to the prelay supplemented group. The unsupplemented group also displayed a significantly higher total calcium concentration at mid and late lay and an increase in ionised calcium between mid and late lay combined with a slower decrease in total phosphorus. Thus the absence of a prelay diet may have led to an increase in osteoid formation earlier in lay due to higher bone calcium reserves.

The provision of a prelay diet appeared to cause a delay in the onset of osteoid formation leading to a deficit in bone mineral at late lay and lower total calcium in the blood stream. This could explain the high percentage of on farm seconds and the incidence of type B's and erosion observed at this time. Despite the apparently beneficial lengthening of the period of high production and earlier onset of high egg weight, the prelay diet has in effect detrimentally altered the skeletal dynamics of the bird.

In terms of bone histomorphometry there were few differences between the two groups. Trabecular bone volume was largely



around the 11% margin indicative of severe osteoporosis in both groups (Whitehead & Wilson, 1992). Although previous authors demonstrated prelay calcium being incorporated into structural bone (Hurwitz, 1964) there was no obvious difference in trabecular bone volume at the beginning of lay in the current study.

In mineral terms although % bone ash remained constant across lay, variation was apparent in % bone phosphorus. The group of hens fed on a prelay diet demonstrated significantly higher % bone phosphorus at mid lay, possibly reflecting poor calcium availability from the bone at this time. Observable changes in the phosphorus concentration were present in both the eggshell and bone at mid lay. Phosphorus is an acknowledged crystal poison and has been implicated in the cessation of mineralisation in the avian egg (Sauveur & Mongin, 1983) leading to a decrease in eggshell strength (Antillon *et al*, 1977). However, the shells under question were not appreciably thinner and the presence of a good cuticular layer on the surface of these shells, with its associated hydroxyapatite, may serve as an explanation for these findings. Alternatively, phosphorus may have been incorporated into the organic matrix of the shell, as previously observed in bone matrix composition (Luck & Scanes, 1979), and the reported results may be indicative of an alteration in eggshell matrix composition.

At mid lay, corresponding to peak ultrastructural eggshell quality, it is reasonable to expect changes in the rate of bone remodelling. In the present situation, this period witnessed a decline in remodelling which was consistent with changes occurring in the eggshell and in the blood. At the end of lay it was clear that such a stress on the system could not be sustained as evidenced by the significant drop in production. This hypothesis is substantiated by the evidence supplied by Bell & Siller (1962)



who demonstrated that the heavy laying modern strains were on the threshold of their minimum endogenous calcium requirements, yet lacked a mechanism to allow lay to cease prior to the onset of osteoporosis. The trabecular bone volumes of both groups were indicative of osteoporosis (Whitehead & Wilson, 1992). The observed increase in the concentration of bone phosphorus suggests a deficit in calcium for incorporation into calcium hydroxyapatite in the bone matrix (Gorski, 1992). Such matrix changes are common in osteoporotic bones due to the necessity for constant bone repair (Knott *et al*, 1995).

In conclusion, the addition of a prelay diet was not reflected in any changes in medullary bone volume or bone mineral composition at early lay as would be expected if the extra calcium provided had been incorporated into medullary bone. The main effect of feeding a prelay diet appeared to be a delay in the increase in osteoid formation, accompanied by a decreased level of ionised calcium in the bloodstream at late lay. "On farm" the changes associated with feeding a prelay diet appeared to be a longer sustained high production rate and earlier increase in egg weight. However this was accompanied by significantly higher "on farm" seconds at late lay due in part to a higher incidence of the ultrastructural variants erosion and type B's. In contrast, however, the indices of fracture toughness and deformation were sustained for longer in the prelay diet group. Although some differences were found between the two groups in this experiment they were not conclusive and no proof was forthcoming to suggest that extra calcium provided in the prelay diet during medullary bone formation led to a better calcium reservoir or a decreased incidence of osteoporosis.

Under commercial conditions the inclusion of a prelay diet continues to be routinely practised as an intermediary between

the starter and layer dietary phases. Recently (BOCM *pers comm*) it has been suggested that the prelay diet reduces the stresses associated with transferring the pullets to the laying house and the subsequent onset of lay. It is clear from this study, however, that further experimentation on a commercial scale is required to clarify its role.

## CHAPTER 3

### OBSERVATIONS ON PIGMENTATION AND ULTRASTRUCTURE IN GUINEA FOWL (*COUSHIELS*)

**CHAPTER 5**  
**OBSERVATIONS ON PIGMENTATION AND**  
**ULTRASTRUCTURE IN GUINEA FOWL EGGSHELLS.**

**CHAPTER 5**

**OBSERVATIONS ON PIGMENTATION AND**  
**ULTRASTRUCTURE IN GUINEA FOWL EGGSHELLS.**

## **5 OBSERVATIONS ON PIGMENTATION AND ULTRASTRUCTURE IN GUINEA FOWL EGGSHELLS.**

### **5.1 INTRODUCTION**

One of the effects of integration with the larger EU market is the appearance of 'exotic' foodstuffs on the supermarket shelf. Thus the guinea fowl is now a readily acceptable addition to the more common place turkey and chicken within the UK. The final chapter of this thesis addresses the commercial problem regarding eggshell quality within the guinea fowl.

Guinea fowl are flock dwelling birds native of both temperate and tropical climates, readily observable on the plains of Africa and India and made popular on the continent by their strong red game-like meat. In recent years the bird has been subject to intensive management conditions facilitated by an immunity to the common viral diseases of conventional fowl.

Lay is known to commence at approximately 28 weeks yielding, on average 150-160 eggs a year (Panda & Mohapatra, 1989). The eggs are strongly pigmented and have an incubation period of 27 days. Although under commercial conditions the majority of eggs fit the pigmented blueprint, unpigmented eggshells are also observed, and are consistently associated with a decrease in hatchability.

Eggshell pigments are characterised by crystalline porphyrins composed of four pyrrole rings. The existence of a central metal ion carrier (Kennedy & Vevers, 1976) is under debate, although Solomon (1991) has reported consistently negative results for metal ions. The site of pigment manufacture is controversial being

of either haematological derivation (Baird *et al*, 1975) or manufactured by the epithelial cells of the shell gland pouch (Solomon, 1991). Guinea Fowl eggs, being mostly brown in colouration, contain the pigment protoporphyrin but may have additional pigments leading to green or blue hues (i.e. the inclusion of cyanoporphyrins). The pigment is secreted by the epithelial cells in the shell gland pouch in the later stages of eggshell mineralisation, with up to 74% of all pigment being deposited in the final 5 hours of mineralisation (Warren & Conrad, 1942). Thus the majority of pigment is located in the outer layers of the true shell (Baird *et al*, 1975) and the cuticle (Schwartz *et al*, 1975). Solomon (1991) suggested that the localised presence of pigment in the eggshell may act as a damper between the calcite crystals thus increasing crack resistance .

The heritability of eggshell pigmentation has been accepted for almost one century (Hurst, 1905; Blow *et al*, 1950; Francesch *et al*, 1997; Jaffe, 1966), however, in 1991, Wei *et al* reviewed the subject and concluded that "design and methodology problems" had lead to many misleading conclusions. They reported that two major autosomal loci were responsible for eggshell colouration, the first controls pigment deposition and has incomplete dominance, whilst the second, is capable of inhibiting pigment deposition only when homozygous recessive. The combination of these two loci could easily be responsible for the wide variety of shell colouration observed.

Heritability of eggshell pigmentation does not predispose consistency either within or between individuals or clutches. The capacity for pigment production reduces with increasing maturity leading to notable changes in colouration (Wells, 1968). More striking however are the changes brought about by stress or disease which lead to hormonal changes within the bird. By far

the largest group of diseases to affect the egg directly are the adenovirus' and egg drop syndrome. Infectious bronchitis, infectious avian encephalomyelitis, turkey rhino-tracheitis and Newcastle disease also have visible effects on pigmentation (Van Ness, 1949; Curtis, 1987). Measures to prevent such diseases may in themselves effect pigmentation changes. Jones *et al* (1990) reported certain anticoccidial agents leading to decreased production, pale eggs and decreased hatchability. Interestingly, eggshell pigmentation proved to be the most sensitive measure of feed contamination by the anticoccidial agent nicarbazin, in a study by Hughes *et al* (1991).

Although changes in eggshell pigmentation are an acknowledged symptom of stress in the avian, there is not yet sufficient evidence to enable its utilisation as a marker of eggshell quality. Both Godfrey (1948) and Campo & Escudero (1984) reported that more highly pigmented eggshells were thicker and stronger than less pigmented eggshells whilst Godfrey (1948) also demonstrated a better rate of hatchability in more highly pigmented eggs, possibly due to lower rates of moisture loss during incubation. Grover *et al* (1980) found a significant relationship between increasing colour intensity of brown eggs and egg specific gravity.

Previous unpublished work on the guinea fowl flock in this study has demonstrated that unpigmented eggshells have a significantly lower eggshell weight and percentage of eggshell than pigmented eggshells (Gautron and Nys *pers comm*). These authors also found that percentage of viable eggs at seven days after the onset of incubation was 95% in the case of the pigmented eggs and 71% in the unpigmented eggs. The reason for the higher mortality during early embryonic development was unclear. The losses at late incubation were similar in both groups. The aims of



the current study were to utilise Scanning Electron Microscopy to examine a possible ultrastructural basis for the observed high mortality during early embryonic development in unpigmented guinea fowl eggshells.

## 5.2 MATERIALS & METHODS

### 5.2.1 ULTRASTRUCTURAL ASSESSMENT OF EGGSHELL

#### QUALITY

Ten pigmented and 10 unpigmented eggshells were supplied, from a mid lay flock of broiler breeder guinea fowl. Ultrastructural assessment was carried out on each egg using the criteria set out for hatching eggs (Figure 92) on the Phillips 501B scanning electron microscope (Philips, Holland).

CONFLUENCE	NONE/ISOL 1	ISOL/MOD 3	MOD/EXT 7		
FUSION	EARLY 10	MAINLY EARLY 8	50:50 3	MAINLY LATE 4	LATE 7
ALIGNMENT	RANDOM 1	MOD. ORGAN 3	FISSURED 5		
TYPE B	NONE/ISOL 1	ISOL/MOD 3	MOD/EXT 7		
DEPRESSION	NONE 1	LIMITED 2	MODERATE 4	DETRIMENTAL 6	
EROSION	NONE 1	LIMITED 3	MODERATE 5	DETRIMENTAL 8	
ARAGONITE	NONE/ISOL 1	ISOL/MOD 3	MOD/EXT 7		
TYPE A	NONE/ISOL 1	ISOL/MOD 3	MOD/EXT 7		
CUBIC	NONE/ISOL 1	ISOL/MOD 3	MOD/EXT 7		
CUFFING	NONE/ISOL 1	ISOL/MOD 3	MOD/EXT 7		
CAPS	GOOD 1	AVERAGE 3	BELOW AVERAGE 5	POOR 7	
CHANGED MEMBRANE	NONE/ISOL 1	ISOL/MOD 4	MOD/EXT 7		

Figure 92: Shell Quality Weighted Score System modified for hatching eggs.

### **5.2.2 ULTRASTRUCTURAL ASSESSMENT OF THE CUTICULAR SURFACE**

1cm<sup>2</sup> samples were removed from the mid line of each eggshell and mounted cuticular layer uppermost on aluminium stubs with conductive silver paint. Samples were then dried in a 60°C oven and coated with gold/palladium prior to scanning electron microscopy. Assessment of the cuticular layer was carried out on a presence or absence basis.

### **5.2.3 STATISTICAL ANALYSIS**

Statistical differences between the experimental groups were analysed using a General Linear Model (glm) for analysis of variance using the Minitab statistical package (Release 11; Minitab Inc, Pasadena, USA).

### **5.3 RESULTS**

#### **5.3.1 ULTRASTRUCTURAL ASSESSMENT OF EGGSHELL QUALITY**

Ultrastructurally the two groups of guinea fowl shells showed no significant differences in terms of total score (Table 18), although the incidence of specific faults was high with respect to confluence, poor cap quality and late fusion as compared with the domestic fowl at mid lay.

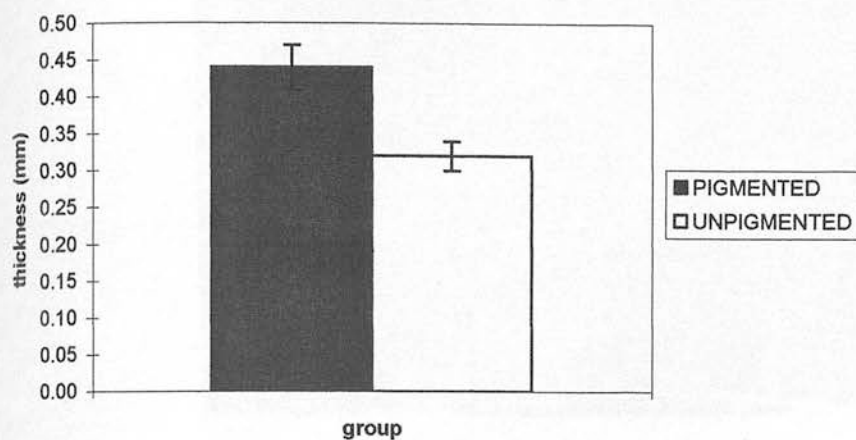
There was a significant difference in both the total thickness (Figure 93) and effective thickness (Figure 94) of the pigmented and unpigmented shells (Figures 95 & 96).

VARIABLE	CONFLUENCE		FUSION		CUFFING		ALIGNMENT		TYPE B		DEPRESSION		EROSION		CUBIC		ARAGONITE	
GROUP	pig	unpig	pig	unpig	pig	unpig	pig	unpig	pig	unpig	pig	unpig	pig	unpig	pig	unpig	pig	unpig
MID (st.dev)	6.70 (0.95)	6.70 (0.95)	6.40 (1.26)	5.50 (1.58)	1.00 (0.00)	1.00 (0.00)	3.20 (0.63)	3.00 (0.00)	3.00 (0.00)	3.40 (1.26)	1.00 (0.00)	1.30 (0.95)	1.20 (0.63)	1.00 (0.00)	1.00 (0.00)	1.00 (0.00)	1.00 (0.00)	1.00 (0.00)
Group	ns		ns		ns		ns		ns		ns		ns		ns		ns	

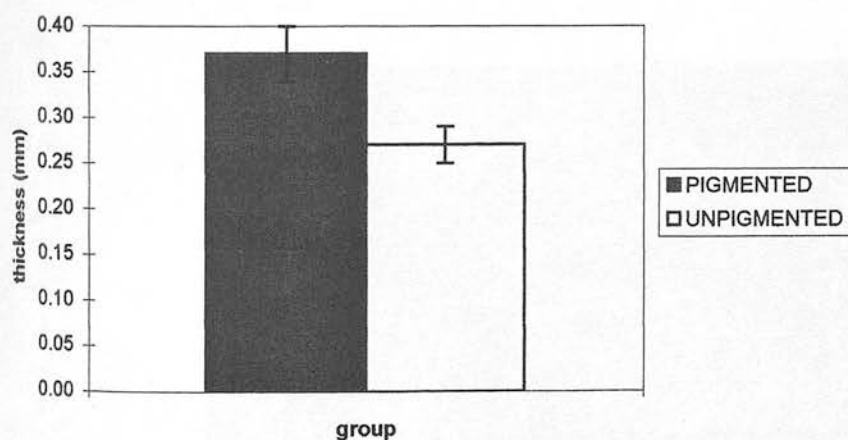
VARIABLE	CAPS		TYPE A		CHANGED MEMBRANE		TOTAL		TOTAL THICKNESS (mm)		MAMMILLARY THICKNESS (mm)		EFFECTIVE THICKNESS (mm)	
GROUP	pig	unpig	pig	unpig	pig	unpig	pig	unpig	pig	unpig	pig	unpig	pig	unpig
MID (st.dev)	6.00 (1.05)	5.20 (2.48)	1.00 (0.00)	1.00 (0.00)	1.00 (0.00)	1.00 (0.00)	32.50 (1.35)	31.10 (4.18)	0.44 (0.03)	0.32 (0.02)	0.06 (0.01)	0.06 (0.02)	0.37 (0.03)	0.27 (0.02)
Group	ns		ns		ns		ns		**		ns		**	

**Table 18:** Influence of group on ultrastructural eggshell quality variables in the laying hen.

() = standard deviation of the mean; \*\*  $p < 0.01$ ; \*  $p < 0.005$ ; ns not statistically significant.

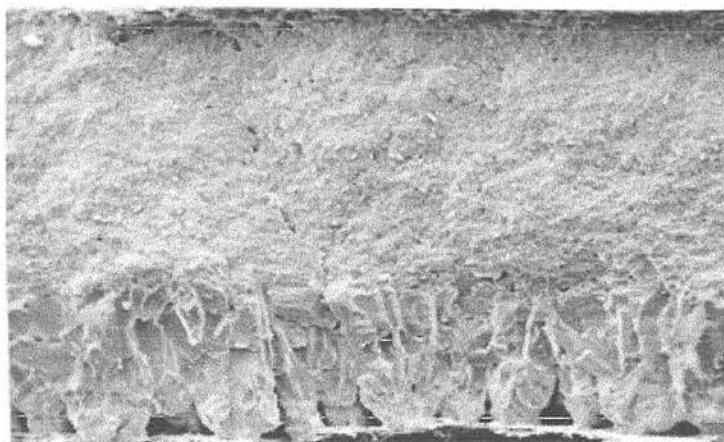


**Figure 93:** Comparison of mean total thickness incorporating standard error of the mean.

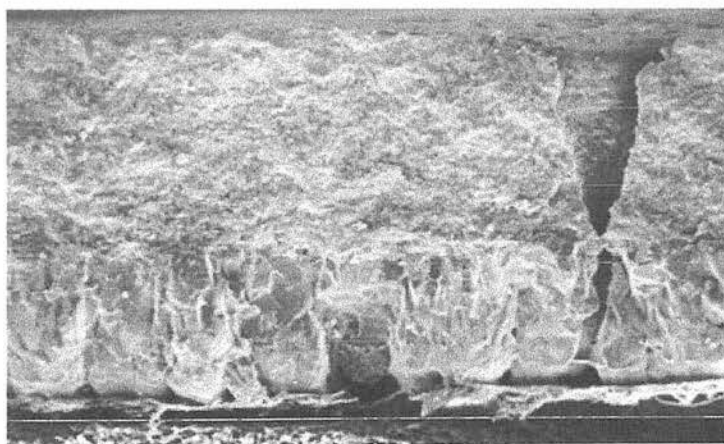


**Figure 94:** Comparison of mean effective thickness incorporating standard error of the mean.





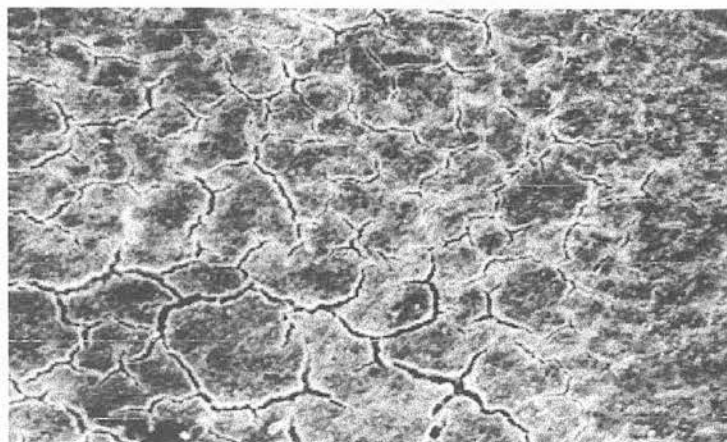
**Figure 95:** A transverse section of a pigmented eggshell demonstrating total, effective and mammillary thickness (x320).



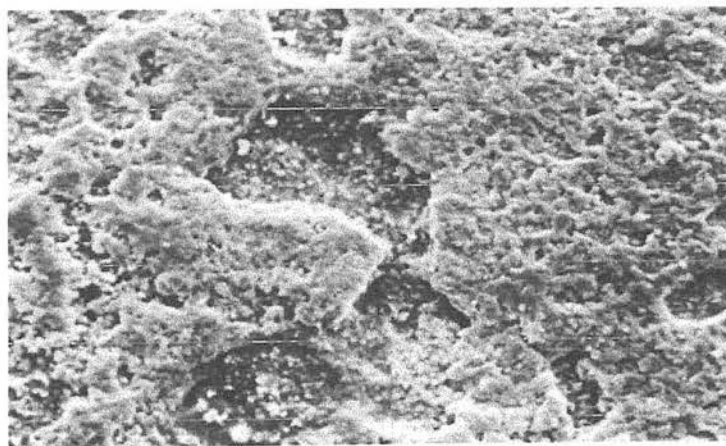
**Figure 96:** A transverse section of an unpigmented eggshell demonstrating total, effective and mammillary thickness (x320).

### **5.3.2 ASSESSMENT OF THE CUTICULAR SURFACE**

All of the sections from pigmented shells showed a good coverage of cuticle with the familiar “cracked mud” appearance (Figure 97) whilst, none of the sections from unpigmented shells showed evidence of a normal cuticle (Figure 98).



**Figure 97:** An electron micrograph of the cuticular layer of the pigmented eggshell demonstrating 'normal' cuticle (x1440).



**Figure 98:** An electron micrograph of the cuticular layer of the unpigmented eggshell lacking the 'normal' cuticle seen in the pigmented shell (x1440).

#### 5.4 DISCUSSION

The age associated decrease in shell colour is evident in the modern commercial laying hen with its high egg output. At the end of lay, these "paler" shells also display a high degree of structural variation within their mammillary layer, leading to elevated total score values of 40 or more (Solomon *pers comm*). Nys & Gautron (*pers comm*) have also reported a correlation between the incidence of unpigmented eggshells and increasing age in commercial guinea fowl flocks.

In 1985 Watt demonstrated an association between time of stress and the position of the egg within the oviduct; thus if the stress event occurred when a fully shelled egg was located in the shell gland pouch, the result was egg retention accompanied by the presence of extra calcium deposits on egg expulsion. If the event occurred at or preceding the early phase of calcium deposition, the mammillary layer presented a variety of structural anomalies commensurate with oviducal breakdown. The fact that both the unpigmented and pigmented shells in the current study were structurally sound at the level of the mammillary layer suggests that the mineralisation process was proceeding unchecked until this time. Given the similarities in the construction of the mammillary layer in both groups, one would have anticipated a similar growth pattern with reference to their respective palisade columns; this was not the case. The palisade columns of the pigmented shells proceeded through the formation of the vertical crystal layer and the cuticle, and thus demonstrated a statistically significant difference in total shell thickness.

An appraisal of the outer surface of the shells revealed that, not only was the cuticle missing from the unpigmented eggshells, but that the vertical crystal layer and part of the outer palisade columns were also absent (see micrographs). Recent data from Fraser *et al* (1999) suggest that at mid lay in domestic fowl, the cuticle makes a major contribution to the total protein content of the shell. Its absence might therefore compromise the fracture mechanics of the shells predisposing them to increased fragility.

In the domestic fowl the withdrawal of calcium from the mammillary layer begins between days 10-12 of incubation (Tuan & Scott, 1977). Nys (*pers comm*) reports a similar time of uptake in the guinea fowl eggshell. The early embryonic death observed in the unpigmented eggshells in this investigation is not consistent with a failure in the structure of the calcium uptake region, in terms of its morphology the mammillary layers of both groups would have served adequately in this process. However, the absence of the outer layers in the unpigmented shells would have created a structurally different embryonic chamber with an increased likelihood of bacterial transfer and a variation in porosity. These two factors alone could account for the significant increase in mortality during the first seven days of incubation in the unpigmented eggshells. Hulet *et al* (1985) demonstrated significantly lower shell thickness values and hatchability in Chinese ring-necked pheasant eggs pigmented tan and blue as opposed to those pigmented olive, dark brown or grey. They attributed the former to a decrease in the time spent in the shell gland pouch.

Although this investigation represents only one moment in time, it is nevertheless the most significant period in terms of shell quality. It has been reported by several authors that shell quality

peaks at mid lay in the eggs of domestic fowl (Reid, 1984; Watt, 1985, Bain, 1990), an observation also corroborated by the study of eggshells from other species (Solomon *pers comm*). It is reasonable to hypothesise, that eggshell quality was at a peak in the guinea fowl at 37 weeks of age. In general hatchability improves to mid lay and thereafter declines and the 95% hatchability observed in the pigmented shells at this time confirms this age associated relationship with shell quality.

Within any one flock the response to stress is not ubiquitous (Watt, 1985) and it is feasible that birds such as guinea fowl which have not been subjected to the pressure of intense selection, may display a greater individual response to stressors. Over the years evidence has accumulated to verify that the eggshell is a valuable non-invasive indicator of the response to stress (Watt, 1985) and the work of Hughes and Gilbert (1984) has provided evidence to correlate changes in the surface morphology of the eggshell with egg retention and premature expulsion in response to noxious stimuli. The quest to identify a stress factor within the commercial environment is fraught with difficulties but invariably involves inadequate management procedures. In the present investigation it was reported that preceding the occurrence of unpigmented shells on farm, the birds had been artificially inseminated. Although this is a routine procedure there is no evidence to show that the process was carried out by the same individuals using identical protocols. Artificial insemination is a fairly aggressive process and might possibly cause uterine injury if incorrectly administered. In view of the fact that the unpigmented egg syndrome increases with bird age (Nys *pers comm*) it is tempting to speculate that birds once physically traumatised and thereafter inseminated will display a greater propensity to oviposit unpigmented eggshells.

## CHAPTER 6

### GENERAL DISCUSSION AND CONCLUSIONS



## 6 GENERAL DISCUSSION AND CONCLUSIONS

The demands placed upon the avian skeleton to satisfy the requirements of the reproductive effort are not unique to commercially grown birds. As a whole, the class Aves lives in a state of calcium compromise in which egg production will always be satisfied at the expense of bone structure and function.

The relationship between the calcium reservoir resident within medullary bone and the fully formed eggshell is neither simple nor straightforward. *In vitro* evidence permits one to visualise a series of interconnecting pathways in which neither bone nor shell is compromised. *In vivo* this rarely happens. The pressure of increased egg output together with environmental variables, such as noise, stocking density and temperature, place considerable demands upon the skeletal system and the endocrine functions of organs such as the pituitary, adrenals, parathyroids and ultimobranchial body.

The processes of bone formation and shell construction share many features in common; thus in addition to the need for the presence of a supersaturated solution of calcium, both require the presence of an organic matrix, which not only facilitates the nucleation of the calcium ions, but exerts a strong influence in terms of rendering both with a degree of flexibility.

This thesis addresses the question of calcium compromise by considering amongst other things the annual cycle of reproductive activity which in the case of the shell, witnesses the introduction of many detrimental effects at the end of lay and at which time bone is, in the common hybrid bird, invariably classified as osteoporotic. If as stated by Solomon (*pers comm*), the shell is

the daily indicator of the bird's harmony with its environment then it is not unreasonable to expect it to reveal to some extent the status of the skeletal system.

The nature of the work involved the use of two main research tools viz. quantitative eggshell analyses and the osteocalcin assay for bone turnover. With reference to the former, many of the structural variations within the mammillary layer occur either between normal mammillae or are a replacement for the norm. In general terms they reduce the bonding between shell and shell membranes. In terms of the table egg this situation leads to an increase in crack formation. With reference to the hatching egg and given the location of these modifications, it is not surprising that during the mechanism of calcium withdrawal which occurs preferentially from the membrane bound tips of the mammillary bodies, they remain unaffected by the process. Resident within the shell at hatching therefore they represent a legacy of the status of the original chamber. If at hatching the incidence of these variations is high then one can surmise some impediment to calcium withdrawal. Integral to the whole investigation was the modification of the existing scoring system for layer eggs to suit the hatching egg. Osteocalcin proved to be a reliable indicator of osteoblast activity and hence bone matrix formation. Used in conjunction with alkaline phosphatase, a marker of bone matrix calcification, the two profiled with a high degree of accuracy, the events occurring at the cellular level in bone.

The comparison of the unselected J.Line with the selected commercial strain underlined the unacceptable demands placed upon the skeletal system in today's hybrids, with both eggshell quality and bone integrity including its mineral composition showing signs of compromise at early lay.

Within the global economy, neither poultry meat nor eggs enjoy stability and so it is with some caution, that producers are willing to invest in expensive additions to existing diets. It is of some surprise therefore that the prelay diet has been so extensively adopted in the absence of apparent rigorous investigation.

The results herein have established that no changes were evident in either bone histomorphometry or mineral content at the beginning of lay due to the addition of a prelay diet. The main differences in bone histomorphometry due to the feeding of a prelay diet appeared to be a delay in the increase in osteoid formation, leading to a longer period of sustained high production and an earlier increase in egg weight. Although these changes would be immediately beneficial from the point of view of the commercial producer, the drawback appears to be a significantly higher incidence of "on farm" seconds at late lay, possibly forcing early depopulation of the flock.

According to Solomon *pers comm*, the shells of all birds display the same range of structural variation at the level of the mammillary lay. Thereafter they differ in terms of the degree of vesicular porosity within the palisade layer and in other physical parameters such as shell thickness, egg shape and egg size. It was not surprising therefore to observe within the foundation layer of the guinea fowl egg, a degree of modification consistent with their avian lineage. The eggs analysed in this short investigation, presented as pale shells. The latter are not the prerogative of the guinea fowl. In recent years, within the UK, there has been considerable debate concerning the aetiology of pale eggs from free range flocks in the South of England. The disappearance of colour coincides with the movement of these birds from the house to pasture in summer. It has been surmised

that the sudden exposure to the hot sun is a stress event and that it has caused premature expulsion of the egg prior to pigment deposition. When the birds are returned to the house pigmentation proceeds as normal. Work in Glasgow has shown that these eggs are structural sound at the level of the mammillary layer.

The foundation layers of the guinea fowl eggshells were similarly well formed, the pale shells were however thinner than expected. In following the blueprint for egg formation, the bird is not only taking into account the construction of the mammillary layer, but must also focus on the distance across which gaseous exchange takes place, i.e. total shell thickness. Extremes in thickness and thinness both introduce problems in terms of the ability of the shell to sustain embryonic growth and it was suggested in this study that in the absence of other disease influences and the presence of normal conditions for incubation, that the thinning of these shells had detrimentally altered the gas exchange mechanism, leading to chick mortality.

With calcium as the core constituent and its utilisation by both bone and shell, this thesis set out to investigate a variety of problems of potential interest to industry. Leaving aside the fact that in most of the experimental protocols, the birds were housed and reared under optimal conditions at the Roslin Institute, the hybrids still displayed overt problems in all areas under examination. If these results are scaled up to the potentially more hostile commercial environment, with birds crowding and more competition for food, then it is easy to understand that as stress escalates, the bird is placed in a situation in which because its skeletal support mechanism is compromised, it is unable to meet the fundamental demands of procreation.

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## APPENDICES

APPENDIX 1: A comparison of the raw materials used in experimental diets

RAW MATERIAL	UNIT	STARTER	LAYER DIET	PRE-LAY DIET
	g/kg	g/kg		
barley meal	g/kg	500	200	250
distilled water	g/kg	16.5	18	18
fish meal	g/kg	10	0	0
crude oil	g/kg	15	7	30
crude fish oil	g/kg	0	10	32
vitamin D <sub>3</sub>	g/kg	0.0	0.0	1
vitamin E	g/kg	2.0	2.5	3.5
vitamin K <sub>3</sub>	g/kg	3	3	2.5
vitamin B <sub>12</sub> (1000 µg/g)	g/kg		100	200
veg. meal oil	g/kg	10	10	0
vitamin A	g/kg	3	3	3
vitamin C	g/kg	300	450	310
vitamin B <sub>6</sub>	g/kg	0	0	30

**APPENDICES**



**APPENDIX 1:** A comparison of the raw materials used in experimental diets.

RAW MATERIAL	UNIT	STARTER	LAYER DIET	PRE-LAY DIET
	S	DIET		
barley meal	g/kg	300	300	293
dicalcium phosphate	g/kg	16.6	19	18
fish meal	g/kg	10	0	0
grass meal	g/kg	15	7	50
limestone flour	g/kg	9	10	82
DL-methionine	g/kg	0.9	0.5	1
mineral mix 5	g/kg	2.5	2.5	2.5
sodium chloride	g/kg	3	3	2.5
soya bean meal (48% CP)	g/kg	310	195	205
vegetable oil	g/kg	10	10	0
vitamin mix 4	g/kg	3	3	3
wheat meal	g/kg	320	450	310
vitamin mix 6	g/kg	0	0	33

**APPENDIX 2:** A comparison of the nutritional variables used in experimental diets.

VARIABLE	UNITS	STARTER DIET	LAYER DIET	PRE-LAY DIET
volume	g/kg	1000	1000	1000
metabolisable energy	MJ/kg	11.78983	12.01511	11.36425
crude protein	g/kg	238.1495	187.2537	183.086
fat	g/kg	29.744	31.319	51.8662
cf	g/kg	35.97	32.845	39.034
starch	g/kg	395.85	45588	373.61
dry matter	g/kg	871.8305	867.6475	882.701
moisture	g/kg	128.165	132.35	0
cystine	g/kg	3.71095	3.14407	0
lysine	g/kg	11.5578	8.0453	8.17514
methionine	g/kg	4.34205	3.24015	3.62705
methionine+cystine	g/kg	8.053	6.38422	6.53747
threonine	g/kg	8.51835	6.55589	6.43886
trypsin	g/kg	3.1762	2.4166	2.34917
calcium	g/kg	10.035	10.225	37.5868
sodium	g/kg	1.632	1.612	1.5175
total phosphorus	g/kg	7.6075	7.5276	7.075
available phosphorus	g/kg	5.389	5.4682	5.1077
linoleic acid	g/kg	11.745	12.408	22.8005

**APPENDIX 3:** Standard management techniques applied to experimental birds.

Cage size and design: All birds were housed in Home Office approved single bird cages (Gridfeed Thornber, Lancashire) measuring approximately 40 x 48 x 78 cm.

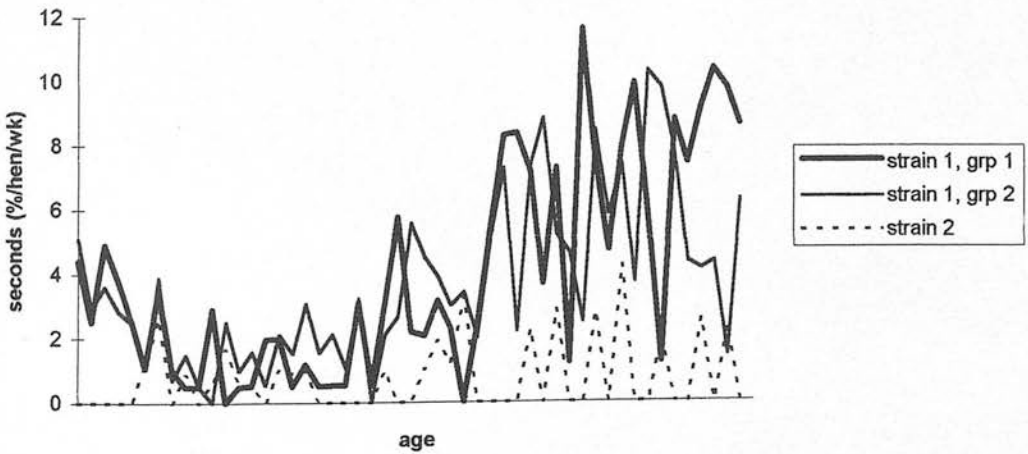
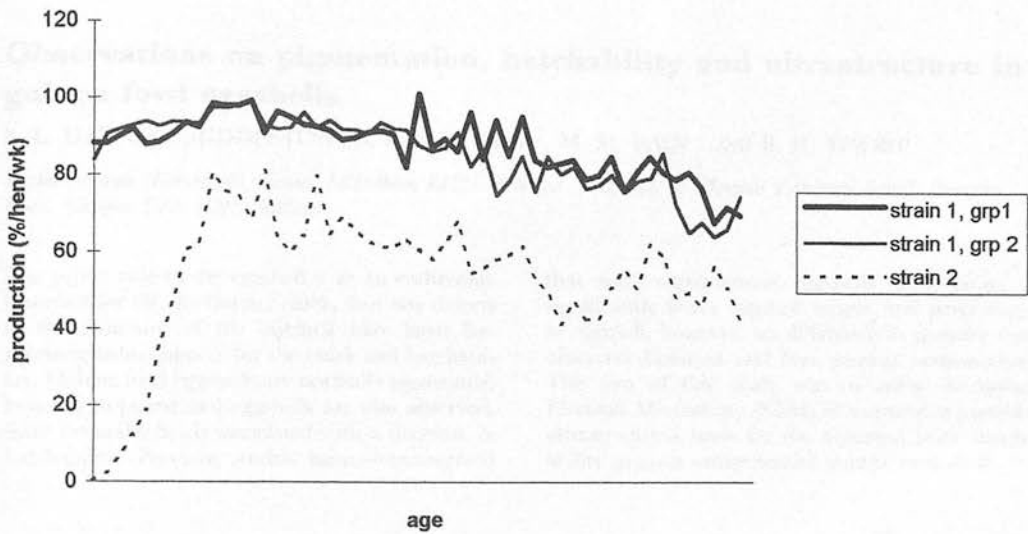
Temperature: 60°C

Water availability: continuous

Lighting programme and intensity: 14 hours light: 10 hours dark,  
250 lux.

APPENDIX 4: A comparison of productivity variables in experimental flocks.

British Poultry Science (1998), 21, 229-233.



**APPENDIX 5: Published work based on this thesis.****British Poultry Science (1998), 39:S28-S29.****Observations on pigmentation, hatchability and ultrastructure in guinea fowl eggshells**

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The prime role of the eggshell is as an embryonic chamber for the developing chick, thus any defects in the structure of the eggshell may have far-reaching consequences for the chick and hatchability. Guinea fowl eggshells are normally pigmented, however unpigmented eggshells are also observed, these normally being associated with a decrease in hatchability. Previous studies have demonstrated

that such unpigmented eggshells demonstrate a significantly lower eggshell weight and percentage of eggshell, however, no difference in porosity was observed (Gautron and Nys, *personal communication*). The aim of this study was to utilise Scanning Electron Microscopy (SEM) to examine a possible ultrastructural basis for the observed poor hatchability in such unpigmented guinea fowl shells.

# APPENDIX 5: Published work based on this thesis (continued).

## British Poultry Science (1998), 39:S28-S29.

MEAT AND EGG SCIENCE

S29

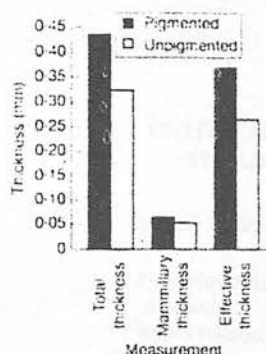


Figure. Assessment of thickness profiles from normal pigmented and unpigmented guinea fowl eggshells.

Ten unpigmented and 10 pigmented shells were supplied. Ultrastructural assessment was carried out on 0.5 cm<sup>2</sup> samples cut from the midline of each egg. The samples had the shell membranes removed by a combination of manual removal and plasma etching (Nanotech 100; Reid, 1983) before being coated with gold/palladium (Sputter Coater SC5000). Mammillary density and the incidence of 12 ultrastructural variants was assessed in each sample, the latter expressed as a total ultrastructural score with reference to the effect on performance of the shell in the field (Reid, 1984; Watt, 1985, 1989; Bain, 1990; Solomon, 1991; Nascimento, 1992). Cuticular coverage was evaluated on 1 cm<sup>2</sup> sections mounted cuticular surface up and coated with gold/palladium. Transverse sections were cut from the midline of the shell, snapped and mounted end on before being coated with gold/palladium. These were analysed to determine the total thickness, mammillary thickness and effective thickness of each sample (Bain, 1990).

There was no significant difference in shell ultrastructure between the pigmented and unpigmented guinea fowl eggs. Both groups displayed a large amount of confluence, along with poor cap quality and late fusion; all features associated with poor ultrastructure. There was, however, a significant difference in both total shell thickness and effective shell thickness (Figure) with pigmented shells demonstrating higher values in both cases ( $P < 0.001$ ). No difference was found in mammillary thickness reflecting a similar time of fusion of the palisade columns. Assessment of the cuticular layer demonstrated that whilst all of the pigmented shells showed good coverage with the familiar 'cracked mud' appearance, none of the unpigmented shells showed evidence of a normal cuticle.

The first prerequisite in the formation of a structurally-sound eggshell is a firm bonding between the soft shell membranes and the first crystals to deposit on to these membranes, that is the mammillary layer. In the absence of this foundation layer, the process of further crystal growth will be impaired. It is apparent, from the many investigations carried out on the eggshell of the domestic fowl, that individual bird response to a stress phenomenon is highly significant. It would appear from this study that all the birds under consideration have, to some degree, experienced a stress effect as indicated by the high incidence of structural faults in the mammillary layer. Nevertheless it is clear that within the group bird responses are not identical.

Thus, in the normal pigmented guinea fowl egg, poor cap quality does not appear to have influenced the formation of subsequent layers of the shell compared with the unpigmented group in which not only has the shell thickness declined, but the birds in question have failed to deposit the outer protective cuticular covering with its complement of pigment. These outermost layers complete the embryonic chamber and are essential for normal embryonic development.

Three theories can be put forward to account for these observations: in response to the stress phenomenon the eggs have been expelled prematurely; under the influence of stress the calcium transfer mechanism and/or the production of the proteins essential for crystal growth has been inhibited; or the birds have been exposed to a disease agent, such as IB. The latter has already been implicated in a reduction in shell colour.

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## APPENDIX 5: Published work based on this thesis (continued).

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### OSTEOCALCIN IN THE LAYING HEN: VARIATIONS WITH STRAIN, AGE AND DIETARY SUPPLEMENTATION

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Bone remodelling and eggshell mineralisation are two calcium dependant processes occurring simultaneously during lay. Under balanced conditions the bird is able to divert calcium to eggshell mineralisation without detrimental effects on bone composition. However under conditions of calcium stress the bird still favours the process of reproduction at the expense of bone remodelling. In the situation where every 24 hours medullary bone is broken down to supply calcium for eggshell mineralisation the bone related effects will change over the laying life as supplies of calcium decrease. In this study osteocalcin was used as a novel marker of medullary bone formation to assess the problem of bone remodelling during the reproductive life of the laying bird. Osteocalcin was assessed using a radioimmunoassay (re Williams, J., Darnell-Middleton, S.L., Nys, Y. & Solomon, S.E. Osteocalcin in the laying hen. Variations during the ovulatory cycle). The levels of calcium, phosphorus, alkaline phosphatase and 1,25 were also assayed to allow clarification of situation. An analysis of the data is presented in the results and discussion

## APPENDIX 5: Published work based on this thesis (continued).

### Proceedings of 10<sup>th</sup> European Poultry Conference, Israel (1998), Volume 2:723-726.



#### VARIATIONS IN PLASMA OSTEOCALCIN AND MINERAL CONCENTRATIONS DURING THE OVULATORY CYCLE OF THE HEN

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#### Summary

An experiment was performed with three groups of 8 laying hens to obtain information on the use of plasma osteocalcin as a marker of bone formation in hens. Blood samples were obtained from hens just prior to and during the period of rapid shell deposition for analysing serum osteocalcin, phosphate and total calcium concentrations. The experimental suppression of egg-shell formation brought about by premature expulsion of the egg resulted in lower plasma phosphate concentrations and a decrease in plasma osteocalcin concentrations compared to the measurements made on control hens given a normal diet. A low calcium diet introduced 24 hours before the experimental sampling period brought about an increase in plasma phosphorus concentrations but did not clearly influence plasma osteocalcin concentrations relative to the control. This was in contrast to previous observations (Nys, 1993) demonstrating that serum osteocalcin increased markedly when hens had been deprived of dietary calcium for a two-week period. Serum osteocalcin can therefore change in relation with mobilisation of medullary bone but further work is needed to validate this marker of osteoblastic activity in hens.

#### Introduction

Osteocalcin, or bone  $\gamma$ -carboxy-glutamic acid protein, is the most abundant non-collagenous protein associated with the mineralised matrix of bone. Its structure and properties were first described by Price (1985) and Hauschka *et al.* (1989) and recently, the complete coding sequence for the chicken 97 amino-acid pre-pro-osteocalcin has been determined (Neugebauer *et al.*, 1995). It is a 5500 Kda  $\text{Ca}^{2+}$  binding protein which contains 49 amino acid residues and three  $\gamma$ -carboxy-glutamic acid residues arising from vitamin K-dependent  $\gamma$ -carboxylation. Osteocalcin is predominantly synthesized by osteoblasts, incorporated into the extracellular matrix of bone but a fraction of the newly synthesized osteocalcin is released into the circulation (Delmas, 1993). Osteocalcin is therefore widely used as a marker for differentiated, mature osteoblasts and clinical osteocalcin assays are currently used in numerous species for monitoring bone formation rates. Its precise function remains unknown but the recent development of osteocalcin deficient mice suggest that osteocalcin may be a negative regulator of bone formation as its absence increases bone formation (Desbois *et al.*, 1996).

At approximately 5 hours after ovulation, the partly-formed egg enters the uterus and at about 10 hours after ovulation, the rapid phase of eggshell calcification commences. This phase lasts approximately 12 hours terminating at around 23 hours following ovulation. The major constituent of the eggshell is calcium carbonate. The calcium necessary is derived in part from the diet since the intestine increases its capacity to retain calcium during the period of shell formation, and in part from mobilisation of skeletal reserves, in particular from the medullary bone which is characteristic of the laying hen and serves as a reservoir to provide calcium for the eggshell. During the period of eggshell calcification, the active resorption surface of medullary bone is increased ninefold and the active osteoblastic surface twofold (Van de Velde *et al.*, 1985).

Given the paucity of data for the domestic hen, variations in the plasma concentrations of osteocalcin throughout the period of egg and shell formation were studied to determine if variations in plasma concentrations may reflect the mobilization of medullary bone. Plasma inorganic phosphorus was also measured as this is classically used as an indicator of bone mobilization. The level of calcium in the diet was one factor that was manipulated for modifying bone mobilisation, and the experimental reduction of the physiological need for calcium by premature expulsion of the partly-formed egg was a further tool used to produce contrasting experimental situations.

## APPENDIX 5: Published work based on this thesis (continued).

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#### Materials & methods

##### Animals and sampling procedures

The experiment utilised three groups of 8 animals taken at random from a flock fed on standard layer mash (36mg Ca<sup>2+</sup>/Kg) and exposed to 14L:10D. Control (C) hens received no treatment, lowCa hens were placed on a low calcium (wheat only) diet 24 hours before the experiment and PG hens received an injection of PGF<sub>2α</sub> (50µg/10µl 0.9% NaCl) at 8 hrs post-oviposition to provoke premature expulsion of the egg. Blood (5ml) was taken at 8, 11, 14 and 17 hours following a mid-sequence oviposition from a wing vein using a heparinised syringe. A further sample 2 hours following oviposition was obtained from a further group of 10 control hens.

Blood plasma was obtained by centrifugation and stored at -20°C until required for assay except for plasma inorganic phosphorus. The times of oviposition were estimated to the nearest minute by means of a trip-switch device fitted to the cages connected to a recording device.

##### Osteocalcin radioimmunoassay

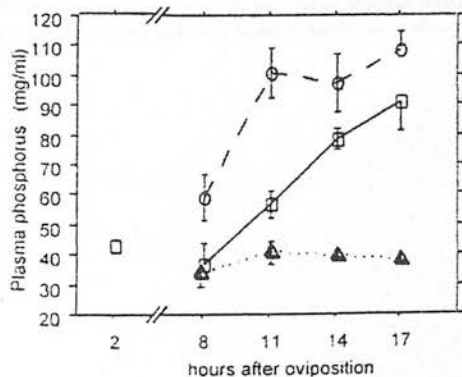
Plasmas were assayed for osteocalcin by radioimmunoassay using pure chick osteocalcin and a specific antibody (KOC1) kindly donated by Dr. R. Bouillon (University of Louvain, Belgium). Briefly, 100 µl standard or sample (diluted 1:10) and 100µl antibody (diluted 1:200,000) were incubated overnight at 4°C and 100µl <sup>125</sup>I-osteocalcin (10,000 c.p.m. labelled using the chloramine-T method) was then added. Following a further overnight incubation at 4°C, 100µl of sheep anti-rabbit serum (1:20) was added and tubes were left to incubate for 2 hrs at 4°C. One ml of a 0.4% PEG 6000 and 0.1% Tween 20 mixture were added and after a final 30 min incubation at 4°C, tubes were centrifuged at 3000g for 30 min and the supernatant was discarded. Bound radioactivity was estimated by counting in a gamma spectrophotometer (Packard Instruments, Meriden, CA, USA) for 1 min. Data were reduced by a computer program based on the 4-parameter logistic method with weighting.

##### Inorganic phosphate assay

Plasma inorganic phosphorus was measured after dialysis of plasma samples on a Technicon autoanalyser.

#### Results

##### Inorganic phosphorus plasma concentrations



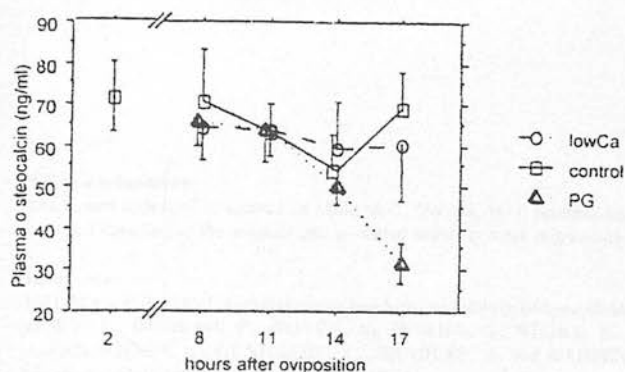
In control hens, the values recorded at 2 hours following oviposition were similar to those recorded at 8 hours after oviposition, then inorganic phosphorus plasma concentrations increased steadily during the period of rapid shell deposition from a mean value of approximately 35 mg/ml at 8 hours following oviposition to a mean value of approximately 90 mg/ml at 17 hours following oviposition. In LowCa hens, ionised phosphorus plasma concentrations also increased from 8 to 17 hours following oviposition, and the mean

concentrations of P were higher than those observed in control hens. These increases were not observed when the egg was prematurely expelled, and the mean concentrations of P in the plasma of PG hens remained low (approximately 35 mg/ml).

# APPENDIX 5: Published work based on this thesis (continued).

## Proceedings of 10<sup>th</sup> European Poultry Conference, Israel (1998), Volume 2:723-726.

### Plasma osteocalcin concentrations



Control hens displayed similar plasma osteocalcin concentrations at 11 & 14 hours after oviposition, ranging from approximately 54-63 ng/ml. The group of control hens sampled at 2 and 17 hours following oviposition displayed higher mean plasma concentrations of osteocalcin (approximately 70 ng/ml). Low Ca hens displayed greater individual variation in the plasma concentrations of osteocalcin and it was not possible to discern any increases or decreases throughout the sampling period. Mean plasma concentrations ranged from approximately 59-64 ng/ml. PG

hens exhibited mean plasma concentrations of osteocalcin of approximately 65 ng/ml at 8 and 11 hours following oviposition, thereafter decreases were observed at 14 hours (mean value approximately 50 ng/ml) and at 17 hours (mean value approximately 32 ng/ml) following oviposition.

### Discussion

The increase in plasma P concentration during eggshell formation reported here for control hens have been observed previously (Mongin and Sauveur, 1979; Nys et al., 1986). These authors also demonstrated that there was no increase in plasma P concentration, hence no medullary bone mobilisation in hens bearing an intra-uterine thread and which laid shell-less eggs. Here, there was also a lack of increase in plasma P concentrations in PG hens whose shell formation was suppressed by premature expulsion of the egg. The absence of eggshell formation clearly suppresses the demand for calcium and therefore there is no bone resorption. In this experimental situation, there was a clear difference in the profiles of plasma osteocalcin concentrations between the PG and the control groups. The mean plasma concentrations were also higher in C hens during eggshell formation. The synthesis of osteocalcin is therefore stimulated by the eggshell formation in agreement with the twofold increase in osteoblastic activity during eggshell calcification (Van de Velde et al., 1985). However, the increase in osteoblastic activity during eggshell calcification did not coincide here with an increase in the concentration of plasma osteocalcin in contrast to previous observations on plasma osteocalcin (Nys, 1993).

Although the low calcium diet resulted in higher plasma phosphate concentrations indicative of greater bone mobilisation, no corresponding increase in plasma osteocalcin concentrations was observed. However, in a previous experiment (Nys, 1993), experimental hens were placed on a low calcium (1.2%) diet for two weeks before blood samples were taken which resulted in a doubling of plasma osteocalcin concentrations and a clear increase during the period of shell deposition. Two conclusions may be drawn from this observation and from the present data. First, 14 hours of a wheat-only diet is sufficient to induce a state of calcium deficiency (judged by the increased plasma phosphorus concentrations) during eggshell formation and secondly, the stimulation of osteocalcin synthesis which depends on 1,25(OH)<sub>2</sub>D<sub>3</sub> production may necessitate more than 7 hours. In contrast, in hens submitted to a longer period of calcium deprivation, there is a large increase in plasma 1,25(OH)<sub>2</sub>D<sub>3</sub> (Nys et al., 1992) and osteocalcin (Nys, 1993). The decrease in osteocalcin in hens laying shell-less eggs and the increase in plasma osteocalcin concentrations in calcium deficient hens suggests that osteocalcin can reflect long term osteoblastic activity. The present observations are not conclusive and additional work is needed to confirm the role of osteocalcin as a bone formation marker in hens.



## APPENDIX 5: Published work based on this thesis (continued).

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